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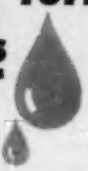

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVII *JANUARY 1958* NUMBER 1

The Antineoplastic Action of Chemical Fractions of the Fruit of *Citrullus colocynthis* on Sarcoma-37*

By R. E. FAUST,† G. E. CWALINA, and E. RAMSTAD

The reported gross and microscopic tumor damage produced by *Citrullus colocynthis* on Sarcoma-37 growing in mice has been verified. The tumor-damaging capacity of the drug has been shown to be intimately associated with the toxic resinous principles of the fruit. Methods for fractionating and studying colocynth constituents, by means of paper partition chromatography, column chromatography, ion-exchange procedures, and dialysis are presented. Two toxic and potent antineoplastic constituents of colocynth resin administered in small doses do not prevent the "take" of S-37. Repeated small doses, however, retard S-37 which is actively growing. Resinous principles producing tumor damage and toxicity are neutral, high molecular weight materials stable to heat.

THE PLANT KINGDOM offers a vast array of chemical compounds and it is not merely optimistic conjecture to postulate that some of these compounds may be valuable in the chemotherapeutic attack on cancer. Indeed, colchicine and podophyllin from the higher plants and bacterial polysaccharides and antibiotics from the lower forms of plant life have already been shown to be active against certain experimental and some human neoplasms.

Belkin and associates at the National Cancer Institute have published a series of papers on the tumor-damaging action of numerous plant preparations, including drugs used as cathartics (1), diuretics (2), and pesticides (3). Fractionation studies have been initiated on several of these drugs in order to ascertain the nature of the components responsible for the antineoplastic activity. When Belkin reported that no such detailed study had been made with *Citrullus colocynthis* (4), we selected this crude drug for our investigation.

Our project was divided into three phases. The first phase involved the development of a suitable technique for the successful transplantation and growth of the experimental mouse tumor, Sarcoma-37, in our laboratories. Next, it was necessary to repeat the preliminary work of Belkin and Fitzgerald (1) in order to demonstrate that our alcoholic extract of colocynth pulp was active against S-37. The final phase was the fractionation of the absolute-alcohol extract of the drug and the search for the components responsible for producing tumor damage.

FRACTIONATION OF THE DRUG

Figures 1, 2, 3, and 4 outline the steps involved to obtain colocynth fractions and constituents by

* Received May 3, 1957, from the School of Pharmacy, Purdue University, Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., New York meeting, April-May, 1957.
† Fellow of the American Foundation for Pharmaceutical Education, American Pharmaceutical Manufacturers' Association Grant. Present address: Potter Drug and Chemical Corp., Malden 48, Mass.

solvent separation, column chromatography, dialysis, and other techniques.

Preliminary Solvent Separations.—Absolute-alcohol extract A was prepared by percolating 75 Gm. of coarsely powdered Colocynth N. F. with absolute alcohol and evaporating the extract *in vacuo* to 20 ml. at a temperature not exceeding 40°. Exactly 500 ml. of absolute-alcohol extract *B* was prepared similarly from 1,000 Gm. of the drug. Exactly 250 ml. of absolute-alcohol extract *B* was evaporated to dryness to give a residue weighing 89.7 Gm. This residue was extracted with chloroform, to give a chloroform-soluble fraction weighing 45.7 Gm. This chloroform-soluble fraction is referred to as the resinous fraction in these studies.

Separation by Means of Column Chromatography.—One gram of the resinous fraction dissolved in 10 ml. of absolute alcohol was added to a column of 25 Gm. of activated alumina. Fractions A–1 (negligible), A-2 (164 mg.), A-3 (590 mg.), and A-4 (86 mg.) were eluted from the column with absolute alcohol, 95% alcohol, 50% alcohol, and distilled water, respectively. One gram of the resinous fraction dissolved in 10 ml. of absolute alcohol was added to a column of 40 Gm. of Florisil. Three distinctly different colored zones were observed: orange-brown, red, and pale yellow. These zones were eluted as fractions F-1 (718 mg.), F-2 (106 mg.), and F-3 (627 mg.) with absolute alcohol, 95% alcohol, and 50% alcohol, respectively. Fraction F-3 contained inorganic salts from the Florisil causing the combined weights of the three fractions to exceed 1 Gm. Because fraction F-2 was subsequently shown to be the most potent fraction, a total of about 0.5 Gm. of it was prepared by the above procedure. Fraction F-2 was further separated into a red water-soluble crystalline material melting at 151–153° and a yellow water-insoluble crystalline portion melting at 125–127°.

A study of the F-2 fraction from the Florisil column demonstrated that the fraction was a mixture of two substances, one giving a yellow color and the other a red color by treatment with phenol and bromine. After separation of the water-soluble and water-insoluble portions of the F-2 fraction, these substances were chromatographed on paper with several different solvent systems and it was concluded that the substances were chromatographically pure.

Separation by Means of Paper Chromatography.—Examination of the resinous fraction by a method related to that described by Mills and Werner (5) showed that it is composed of at least four substances which are detectable by this procedure. Two of these resin components were isolated in large quantity by the following procedure. Each of four large sheets of Whatman No. 1 filter paper (46 × 57 cm.) were streaked at the starting line with 133 mg. of the resinous fraction. The sheets were developed in a large jar equipped for descending chromatography with a solvent system composed of the lower phase of amyl alcohol 7.5 parts, distilled water 4.0 parts, and odorless kerosene 1.0 part. After developing for six to eight hours, the sheets were air-dried. Examination under ultraviolet light revealed a white fluorescent zone near the solvent front, a yellow zone in a band 2–8 cm. from the starting line, and some residual substances at the starting line. When

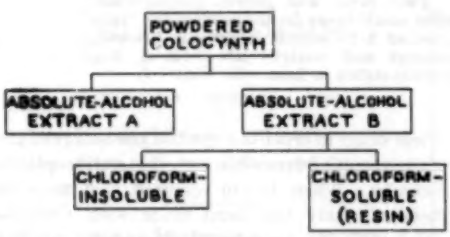

Fig. 1.—Preliminary solvent separations.

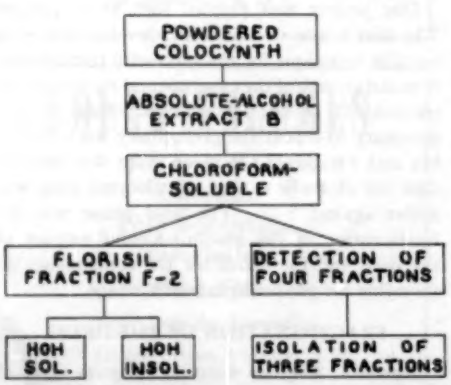

Fig. 2.—Studies with column chromatography.

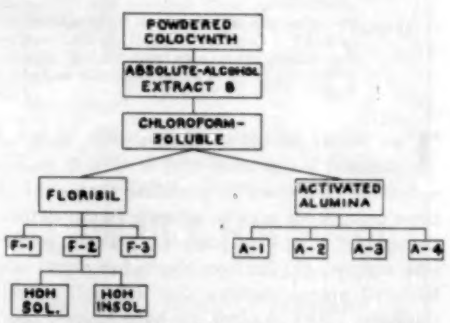

Fig. 3.—Studies with paper chromatography.

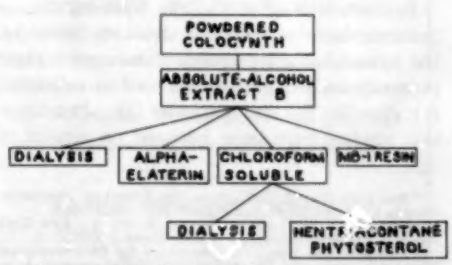

Fig. 4.—Isolation of known constituents and further studies.

a longitudinal strip of the original chromatogram was sprayed with a 50% aqueous phenol solution followed by exposure to bromine vapors, the yellow zone could be located. The areas of the chromatograms containing the components producing the white fluorescence and the yellow zone, as well as the material on the original streak, were cut out and eluted with absolute alcohol. The eluates were then evaporated to dryness over calcium chloride in a vacuum desiccator.

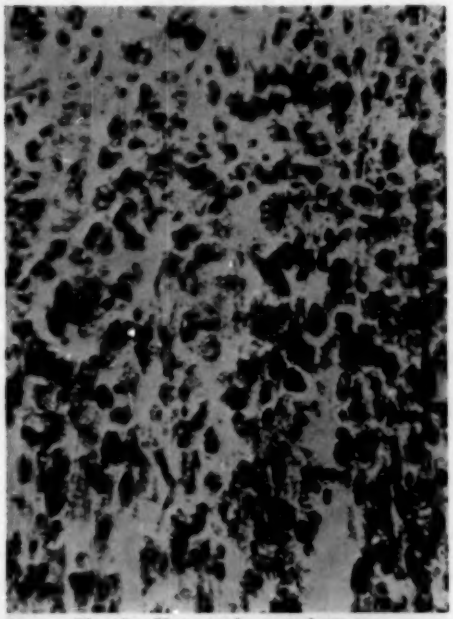

Fig. 5.—Untreated control tumor.

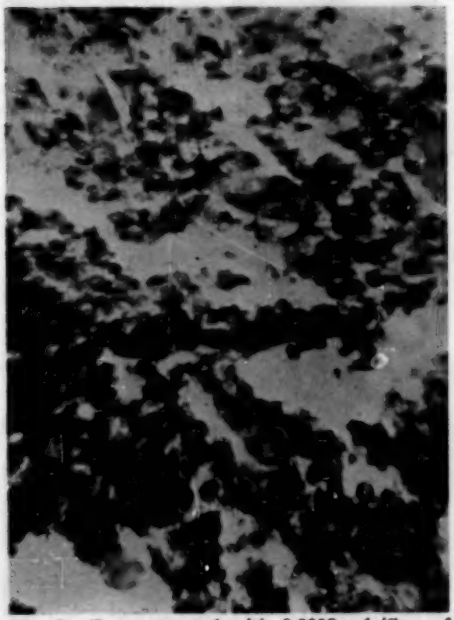

Fig. 6.—Tumor treated with 0.0002 ml./Gm. of absolute alcohol extract A.

Isolation of Other Constituents and Further Separation Studies by Ion-exchange and Dialysis.—A crude fraction containing hentriacontane and phytosterol was prepared following a procedure described by Power and Moore (6). Another fraction containing alpha-elaterin also was prepared (6).

Two ion-exchange columns were prepared with 2.5 Gm. of MB-1 resin.[1] Exactly 2.5 ml. of absolute-alcohol extract *B* was diluted to 50 ml. with absolute alcohol and passed through the two columns. The neutral eluates were collected and evaporated to 12.5 ml.

Dialysis experiments were patterned after one described by Loran and Towbin (7). Fifteen milliliters of 0.01 *M* borate buffer (pH 8.8) was added to 15 ml. of absolute-alcohol extract *B* to give a cloudy suspension. The mixture was placed in a cellophane bag and dialyzed with cold running water for fourteen days. During the first three to four days the diffusate was colored a deep orange-yellow. After filtration and centrifugation, the slightly cloudy dialysate measured 96 ml. and had a pH of 7.5. Exactly 48 ml. of this dialysate was evaporated to dryness in a vacuum desiccator and then suspended in 10 ml. of water. Then, 2.742 Gm. of the resinous fraction, equivalent to 15 ml. of absolute-alcohol extract *B*, was solubilized in 15 ml. of absolute alcohol. The solution was mixed with borate buffer and dialyzed as described. The prepared dialysate (75 ml.) was evaporated to 3 ml.

TESTING AGAINST SARCOMA-37

Preparation and Use of the Test Tumors.—Mice used in this study were female Swiss or CAF_1. Sarcoma-37 was the only neoplasm used and animals bearing the tumor were obtained either from the Jackson Memorial Laboratory or through the courtesy of Dr. M. J. Shear of the National Cancer Institute.

Of several techniques studied the use of a glass tissue homogenizer provided the easiest and most effective method of preparing sterile normal saline suspensions of S-37 cells. During early experiments the tumor suspensions were tested for sterility with fluid thioglycollate medium. Test tumors were prepared by injecting the sterile tumor suspensions intramuscularly into the thigh region. The tumor was maintained by subcutaneous transplantation into the right axillary region of two to four animals every six to ten days.

Comparative histological examinations of treated and control tumors were made in order to evaluate microscopic tumor damage and to ascertain the relationship between gross and microscopic evidence of antineoplastic activity. When tumor tissue was fixed in Zenker-formol solution and stained with hematoxylin-eosin, distinct differences similar to those reported by Belkin and Fitzgerald (1) were noted in the control and treated tumors, as illustrated in Figs. 5 and 6. Figure 7 shows a control

[1] Rohm and Haas mixed bed resin consisting of anionic and cationic exchange resins.

tumor and Fig. 8 a treated tumor which is necrotic and hemorrhagic. Since there appeared to be a close relationship between microscopic tumor damage and the gross observation of hemorrhage and necrosis, evaluation of tumor damage was based principally on gross observation of differences between treated and control tumors. The following code was used to describe the gross appearance of tumors: 0—no hemorrhage or necrosis, 1—slight hemorrhage and necrosis, 2—moderate hemorrhage and necrosis, and 3—severe hemorrhage and necrosis.

Drug fractions were administered subcutaneously into the flank contralateral to the thigh tumor. Animals were usually sacrificed twelve, twenty-four, forty, or forty-eight hours after administration of the drug. The weights of the tumor and spleen of many treated and control animals were recorded.

Effect of Absolute Alcohol Extracts *A* and *B*.—Studies with 39 CAF_1 and Swiss mice, which are summarized in Table I, showed that doses of 0.0002 ml. of the extract per gram of animal caused moderate to severe damage to tumors growing subcutaneously or intramuscularly. Toxic symptoms

TABLE I.—EFFECT OF ABSOLUTE ALCOHOL EXTRACTS *A* AND *B* ON S-37

Extract	Control		Treated	
	Wt.	Effect	Wt.	Effect
A	2.634	0	1.654	+2 to +3
B	3.007	0	2.017	+2 to +3

observed in these and other experiments included weight loss, diarrhea, intestinal hyperemia, depression, and extensive brownish yellow discoloration and irritation at the area of drug release. Higher doses resulted in death within the forty-eight-hour experimental period. Drug fractions were dispersed in methyl cellulose solution or dissolved in propylene glycol.

Effect of Chloroform-Soluble and Chloroform-Insoluble Fractions.—Both fractions produced tumor damage with concomitant toxic symptoms, but these effects were predominant in the animals injected with smaller doses of the chloroform-soluble fraction.

Effect of Fractions Obtained from Column Chromatography.—Fractions A-2 and A-3 were tumor-damaging and toxic, while fraction A-4 produced no tumor damage. Fractions F-1 and F-2 caused the usual toxic symptoms as well as severe tumor damage. However, the F-3 fraction was nontoxic and possessed no activity against the tumor. Table II summarizes the effect of repeated doses of the F-2 fraction on tumor "take." On the first, second, third, and fifth day following tumor inoculation, the ten animals in the treated group received fraction F-2 dissolved in propylene glycol in doses of 0.035 mg. per Gm. at each injection. The animals were sacrificed on the eighth day of tumor growth and the tumors examined and weighed. Ten control animals received injections of propylene glycol. All animals showed no toxic symptoms, but did have irritation at the area where the drug was injected. Table III summarizes the effect of repeated doses of fraction F-2 on S-37 growing actively in the mouse. On the fifth, sixth, seventh, and ninth day of tumor growth eleven animals received a dose

TABLE II.—EFFECT OF REPEATED DOSES OF FRACTION F-2 ON TUMOR "TAKES"

	Av. Wt., Gm.		Av. Tumor Wt., Gm.	
	Control	Treated	Control	Treated
At first injection	20.6	21.1	...	...
When sacrificed	22.4	20.4	2.487	1.923

Fig. 7.—Untreated control tumor.

Fig. 8.—Tumor treated with 0.0007 ml./Gm. of absolute alcohol extract B.

TABLE III.—EFFECT OF REPEATED DOSES OF FRACTION F-2 ON ACTIVELY GROWING S-37

Age of Tumor, Days	Av. Animal Wt., Gm.		Av. Tumor Diam., mm.	
	Control	Treated	Control	Treated
5	20.6	21.2	7.9	8.1
7	21.8	23.9	12.1	11.3
9	21.3	20.7	15.0	14.6

	Av. Tumor Wt., Gm.	
	Control	Treated
At sacrifice (9 or 11 days)	3.179	2.815

of 0.035 mg. of F-2 per Gm. Eleven animals served as controls. All animals were sacrificed either on the ninth or eleventh day of tumor growth, the tumors examined for evidence of damage, and the weights of the tumor and spleen recorded. All treated animals showed mild toxic drug symptoms and irritation at the site of drug release. Upon autopsy all treated animals had tumors which were damaged from a moderate to severe degree.

Of the six resin fractions tested against S-37 the F-2 fraction was the most toxic and produced tumor damage at the lowest dosage levels. The water-insoluble portion obtained from the F-2 fraction was found to be far more toxic and deleterious to the tumor than the water-soluble portion.

Effect of Fractions Obtained by Paper Chromatography.—The fractions corresponding to the material on the original streak and to the resin component giving a yellow color in ultraviolet light were very toxic and produced tumor damage. The fraction corresponding to the substance producing a white fluorescence caused mild diarrhea and other toxic symptoms, as well as slight tumor damage.

Effect of Known Plant Constituents and Material Obtained by Ion Exchange and Dialysis Studies.—None of the animals treated with the fraction containing alpha-elaterin or with the fraction of hentriacontane and phytosterol showed tumor damage or toxic symptoms. Doses of the concentrated eluate from the MB-1 columns ranging from 0.0017 to 0.01 ml. per Gm. produced toxic symptoms and moderate to severe tumor damage. Doses of the concentrated dialysate of absolute-alcohol extract *B* and of the resinous fraction ranging from 0.007 to 0.017 ml. per Gm. also produced the familiar toxic symptoms and tumor damage. In several instances severe tumor damage was accompanied by only mild toxic symptoms.

Effect of Heat on the Activity of the Resin.—Heating a propylene glycol solution of the chloroform-soluble fraction for fifteen minutes at 100° does not alter or destroy its capacity to produce tumor damage in experimental animals. The tumor damage is still associated with toxic symptoms.

The Effect of Antihistamine on the Action of Colocynth Fractions.—Barrett (8) has reported the tumor-damaging effects of histamine. Our preliminary studies showed that a dose of 10 mg. of histamine base (as histamine dihydrochloride) produced severe hemorrhage and necrosis in S-37. The tumor damage was grossly indistinguishable from that caused by various colocynth fractions and was accompanied by only mild toxic symptoms, chiefly depression. Since the release of histamine within the animal due to the irritation of the drug may be responsible for the damage to the tumor, an experiment was designed in which an antihistamine was injected prior to the drug. Promethazine hydrochloride N. F. was injected intraperitoneally at doses of 0.01 mg. per Gm. thirty minutes before injection of either the water-insoluble portion of the F-2 fraction or the resinous fraction. The antihistamine at this concentration did not prevent tumor damage or toxic symptoms.

SUMMARY

1. Over 400 Swiss and CAF_1 mice bearing S-37 growing subcutaneously or in the thigh muscle were used to study the antineoplastic effect of numerous colocynth fractions prepared by solvent fractionation, paper chromatography, column chromatography, ion exchange techniques, and dialysis.

2. One of the prominent observations of the investigation is that the tumor-damaging capacity of the active colocynth fractions appears to be intimately associated with the toxic principles of the drug, chiefly the resin.

3. A potent tumor-damaging and toxic portion of the resin (F-2 fraction) eluted from a Florisil column does not prevent the "take" of Sarcoma-37 when administered in small repeated doses, but does slightly retard its growth. Also, repeated doses of this fraction slightly retard the growth of actively-growing S-37 without producing severe toxic manifestations.

4. By fractionation on a Florisil column, two chromatographically pure constituents of colocynth resin have been isolated, one melting at 126° and the other at 152°. Each of these is toxic and tumor-damaging.

5. Crude fractions of hentriacontane, phytosterol, and alpha-elaterin, three nonpurgative principles of colocynth, are not tumor-damaging.

6. From ion exchange and dialysis studies some of the colocynth constituents producing tumor damage and toxic reactions appear to be neutral, high-molecular-weight principles.

7. Histamine injections cause tumor damage grossly similar to that produced by various colocynth fractions. Pretreatment of animals with an antihistamine fails to prevent toxic symptoms and damage to the tumor caused by colocynth.

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Certain Metallic Soap-Petrolatum Ointment Bases*

By ROBERT E. SINGISER†‡ and HAROLD M. BEAL‡

Eight metallic soaps have been used to produce ointment bases by gelling the following vehicles: (*a*) white petrolatum, liquid petrolatum, anhydrous lanolin; (*b*) white petrolatum, liquid petrolatum, and (*c*) liquid petrolatum. The bases were studied at 8°, room temperature, and 45°. Penetration results indicate that gels of metallic soaps with heavy liquid petrolatum produced the most desirable preparations. Consistency changes were very slight over the above temperature extremes. These ointment bases are superior to white petrolatum both in thermo-stability and elegance. Anhydrous lanolin in these newly developed bases provides water-absorption capacity with no incompatibility. These new metallic soap gels proved to be superior or equal to other liquid petrolatum gels currently advocated as ointment bases. One hundred seventy-six ointments were prepared for compatibility observations. They represent eleven different medicaments tested with the various soap gels. A vast majority of the ointments prepared from metallic soap bases did not show the loss of homogeneity associated with white petrolatum base ointments. With the proper selection of the metallic soap gel ointment base, any of the eleven medicaments studied can be successfully compounded.

IN SPITE OF THE INTRODUCTION of many new ointment bases, oleaginous bases still enjoy more widespread usage than any other. Since the adoption of petrolatum by the U. S. P. in 1882, few improvements were made on these bases until recent years.

Petrolatum base ointments always become less viscous on storage at elevated temperatures or in warm climates. This often results in syneresis or complete separation of any medicament from the base. Conversely, petrolatum bases become very stiff at low temperatures.

Hydrogenated oils, sulfonated oils, sulfonated-hydrogenated oils, and phosphated-hydrogenated oils have been offered as improved oleaginous bases. Piero (1) stated that 2 per cent 12-hydroxystearic acid will gel mineral oil and produce a liquefying ointment. More recently polyethylene has found considerable use in gelling mineral oils (2, 3). All of these bases are claimed to have good thermal stability. The lubricating industry has long used metallic soap-gelled petrolatums as heat stable lubricants.

The purpose of this investigation was to determine the usability of metallic soap-petrolatum gels as thermally stable ointment bases. A series of metallic soaps, in three different bases, was employed to ascertain their relative efficacy and their compatibility with various medicaments commonly used in oleaginous ointment bases. These metallic soap-gelled petrolatum ointment bases were compared to white petrolatum and petrolatum bases gelled with 12-hydroxystearic acid and polyethylene.

EXPERIMENTAL

Ointment Bases Studied

Selection of Metallic Soaps.—Preliminary testing was carried out on 25 samples of metallic soaps, representing 11 different soaps. On the basis of the relative gel strengths and appearance, eight of these samples were felt to warrant further investigation.

Formulas of Bases.—Table I shows the formulas which were employed with each of the soaps. Generally, each soap was prepared in three different bases. These ointment bases are numerically arranged so that: (*a*) even hundred bases (100, 200, 300, etc.) consist of white petrolatum, U. S. P. and heavy liquid petrolatum, U. S. P.; (*b*) quarter-hundreds (125, 225, 325, etc.) indicate a base of heavy liquid petrolatum, U. S. P.; and (*c*) half-hundred bases (150, 250, 350, etc.) consist of white petrolatum, U. S. P., heavy liquid petrolatum, U. S. P., and anhydrous lanolin, U. S. P.

Preparation of Bases.—Approximately three-kilogram size batches of the base were prepared, using thermal dispersion procedures widely used in the lubricating grease industry (4–8). Either a slow cooling process involving an eight-hour annealing at 70° and subsequent slow cooling to room temperature, or a shock cooling procedure was used in preparing these bases.

All of the bases were milled a total of three times by passage through a three-roll mill with roll clearances of 0.002 inch.

Evaluation.—All of the bases shown in Table I were packed in one-pound ointment jars and stored as follows: (*a*) at 6–8° in an electric refrigerator, (*b*) at room temperature, and (*c*) at 45° in an electric oven. These ointment bases were evaluated primarily by measuring their consistency. The method used was ASTM Method D 217–52 T (9), employing a penetrometer equipped with a total movable plunger assembly weighing 150.0 Gm. The penetration results given herein are in tenths

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TABLE I.—OINTMENT BASES STUDIED WITH VISUAL OBSERVATIONS AFTER TWO MONTHS STORAGE

Base No.	Gelling Agent	Soap, %	Liquid Petrolatum, %	White Petrolatum, %	Lanolin Anhydrous, %	Dispersion Temp. °C.	Cooling Method	Storage Temperature 8°	RT	45°
60	Polyethylene[a]	..	q. s.	...	...	...	...	T-1, S, v. sl. B	T-1, S	T-2, sl. T-6, S, sl. B
75	.....	..	..	100.0[b]	...	...	...	T-3, T-5	T-3, sl. T-5	T-2, T-3
80	.....	..	..	100.0[c]	...	...	...	T-3, sl. T-5	T-3, v. sl. T-5	T-2, T-3
100	Al Stearate (Mono) GM[d]	12.0	43.0	45.0	...	95	Slow	T-2	T-2	T-2
125	Al Stearate (Mono) GM	14.0	86.0	...	...	100	Slow	T-1	T-1	T-1
150	Al Stearate (Mono) GM	12.0	42.0	40.0	6.0	95	Slow	T-1	T-1, WA(14%)	T-1
200	Al Stearate G-100, Aero[e]	7 0	46.0	47.0	...	105	Slow	T-2	T-2	T-2, v. sl. B
225	Al Stearate G-100, Aero	7.0	93.0	...	...	105	Slow	T-2	T-2	T-2, sl. B
250	Al Stearate G-100, Aero	7.0	45.0	42.0	6.0	110	Slow	T-1	T-1, WA(11%)	T-1, v. sl. B
300	Al Palmitate[d]	12.0	42.0	46.0	...	115	Slow	T-1	T-1	T-1
325	Al Palmitate	14.0	86.0	...	...	115	Slow	T-1	T-1	T-2, sl. T-6
350	Al Palmitate	12.0	42.0	40.0	6.0	115	Slow	T-1	T-1, WA(10%)	T-1
400	Al Laurate #10197[f]	[g]								
425	Al Laurate #10197	14.0	86.0	...	...	130	Slow	[h]	T-2	T-2, sl. T-6
450	Al Laurate #10197	12.0	42.0	40.0	6.0	130	Slow	T-1	T-1, sl. S, WA (16%)	T-1, v. S, sl. B
500	Li Stearate #306[i]	10.0	43.0	47.0	...	200	Slow	T-1	T-1	T-1
525	Li Stearate #306	12.0	88.0	...	...	220	Slow	T-1	T-1	T-1
550	Li Stearate #306	10.0	42.0	42.0	6.0	200	Slow	T-1	T-1, WA(12%)	T-1
600	Li Hydroxystearate[i]	10.0	47.0	43.0	...	220	Slow	T-2	T-2	T-2
625	Li Hydroxystearate	12.0	88.0	...	...	220	Rapid	T-2	T-2	T-2
650	Li Hydroxystearate	10.0	44.0	40.0	6.0	220	Slow	T-1	T-1, sl. S, WA (9%)	T-1
700	12-Hydroxystearic Acid[j]	5.0	47.0	48.0	...	110	Slow	T-2	T-2	T-2
725	12-Hydroxystearic Acid	9.0	91.0	...	...	110	Slow	T-2, S	T-2, sl. T-5, S	sl. T-5, sl. S
730	12-Hydroxystearic Acid	2.0	98.0	...	...	110	Slow	T-5, B, S	T-5, B, S	T-6, B, S
750	12-Hydroxystearic Acid	5.0	47.0	42.0	6.0	110	Slow	T-2	T-2, WA(6%)	T-2
800	Sodium Stearate[k]	10.0	47.0	43.0	...	260	Rapid	T-1	T-1	T-2
825	Sodium Stearate	12.0	88.0	...	...	260	Rapid	T-2, sl. B	T-2, sl. B	T-2, B
850	Sodium Stearate	10.0	44.0	40.0	6.0	260	Rapid	T-1	T-1, WA(24%)	T-2

Key: v.—very; sl.—slightly; B—bleeding; D—discoloration or color change; NC—no apparent change; P—pourable; S—soft; St—stiff; SL—separation into layers; SM—settling of medicament; T—texture; T-1—buttery; T-2—smooth; T-3—fibrous, stringy; T-4—tacky; T-5—grainy; T-6—lumpy; T-7—gummy; WA(%)—water absorption capacity and approximate w/w% of water held.

[a] Jelene, Research Products Corporation, Madison, Wis.
[b] Snow White Sherolatum, Sherwood Refining Co., Inc., Englewood, N. J.
[c] White Fonoline, L. Sonneborn Sons, Inc., N. Y. C.
[d] Metasap Chemical Co., Harrison, N. J.
[e] American Cyanamid Co., New York, N. Y.
[f] Harshaw Chemical Co., Cleveland, O.
[g] Insufficient soap sample, base not prepared.
[h] Insufficient soap sample, this storage temperature not studied.
[i] Witco Chemical Co., New York, N. Y.
[j] The Baker Castor Oil Co., New York, N. Y.
[k] Fisher Scientific Co., New York, N. Y.

of a millimeter; the unit is not included in the penetration result. The maximum penetration obtainable is 450. Only "unworked" penetration was measured; that is, penetration of sample that received no handling prior to testing nor was it subjected to the action of a "grease worker."

The consistency determinations with the penetrometer were found to yield good comparative results when used as directed by ASTM Method D 217–52 T. Kostenbauder and Martin (10) found the penetrometer yielded poor results except for very hard products. Only in very soft ointment bases, where the penetration was 400 or greater, were the results obtained in this study possibly in error. Here the rate of the falling cone was so rapid that the human error in measuring the five-second free-fall interval became a factor. Figures 1 through 6 show penetration results attained on some of the ointment bases studied.

In order to evaluate the thermal stability of these ointment bases, consistencies were determined at the storage temperatures previously mentioned. To establish a base line for comparison, the penetration on all samples was also determined at 35°. Penetration of the bases at all three storage temperatures was recorded every fifteen days for a two-month period. Immediately following each of these fifteen-day tests all of the samples were placed

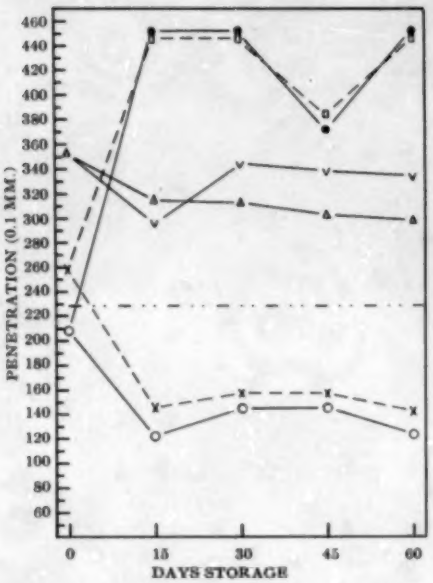

Fig. 1.—Consistency of ointment bases as determined by penetration measurements. Base 60: Δ—at 8°; v—at 45°. Base 75: ○—at 8°; ●—at 45°. Base 80: x—at 8°; □—at 45°—·—·—line is average penetration of white petrolatum, U. S. P. at room temperature.

for a minimum of twenty hours in an electric oven set at 35°; then the penetration tests were repeated.

The ointment bases were observed for visual physical changes each time consistency determinations were made on them. The appearance of each was noted for discoloration, oil separation, texture, settling of medicaments, and any other noticeable change. Table I notes these visual observations.

If as little as one-half per cent of water is present when these metallic soap-petrolatum gels are formed, often the gel is either weakened or it will not form. For this reason the effect of water added to adsorption-type bases made with these gels was observed. Water was incorporated into each of the lanolin-containing ointment bases (half-hundred bases) after it was cooled and milled. The bases were examined for breakage; then stored in tightly closed ointment jars for six weeks and then re-examined. Table I shows the effects of this water-addition.

Ointment Studies

In order that some initial indication of the compatibility of these new bases could be obtained, eleven different ointments were prepared using sixteen different bases.

Formulas of Ointments Prepared.—The hundred and half-hundred series ointment bases of each metallic soap were combined with the various medicaments. For comparative purposes ointments were also prepared using Base 75. The medicinals added to the ointment bases were those commonly found in petrolatum ointments; the concentrations

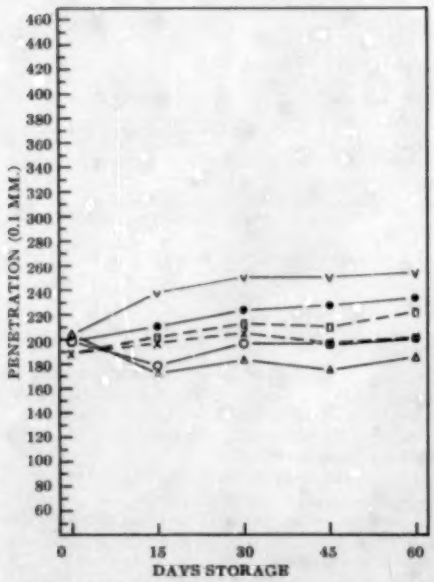

Fig. 2.—Consistency of ointment bases as determined by penetration measurements. Base 125: Δ—at 8°; v—at 45°. Base 325: ○—at 8°; ●—at 45°. Base 425: x—at RT; □—at 45°.

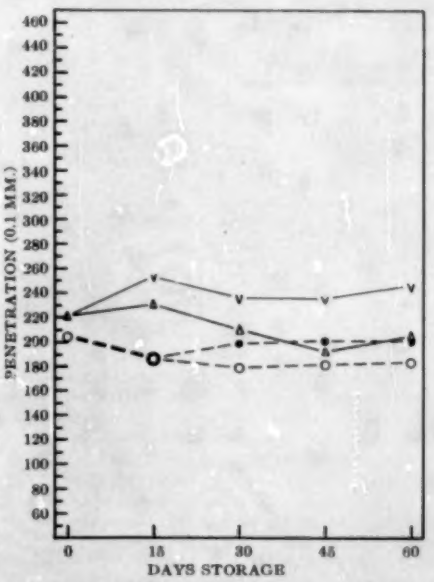

Fig. 3.—Consistency of ointment bases as determined by penetration measurements. Base 525: Δ—at 8°; v—at 45°. Base 625: ○—at 8°; ●—at 45°.

employed were those specified in the official compendia. Table II shows the ointments prepared for this compatibility study. Standard pharmaceutical techniques were used in incorporating these ingredients with the ointment bases. They were finally milled to assure homogeneity.

Evaluation.—Each of the ointments of Table II was stored at 45° in a ten-dram Kimble opticlear vial with a tight cork closure. Visual observations were made on each of the ointments every thirty days for a two-month period. Table II shows these observations. The symbols used in this table are the same as those used in Table I.

DISCUSSION

Structure.—The structure of metallic soap-thickened mineral oil gels has been under intensive investigation for many years by the lubrication industry. Many factors contribute to the final structure of the gel; however space limitations prevent a satisfactory discussion of these factors. C. J. Boner (4) has made an exhaustive compilation of the modern views regarding gel structure.

From the work reported, it is apparent that the gels contain a colloidal fiber structure, the fiber type being characteristic of the soap employed. The most desirable fiber form for shear stability is one with a high length-to-diameter ratio. The best fiber form for holding the oil firmly is one with a low ratio. From the viewpoint of use as ointment bases, it would seem short fibered products would be best. Shear breakdown due to inunction would actually be desirable; small fibers would provide this and at the same time hold the oil firmly to prevent syneresis.

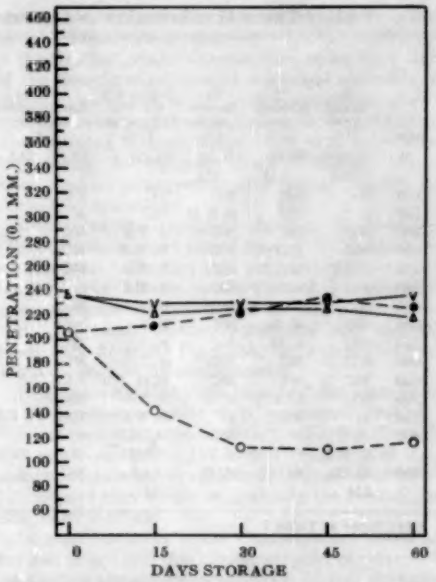

Fig. 5.—Consistency of ointment bases as determined by penetration measurements. Base 825: Δ—at 8°; v—at 45°. Base 850: ○—at 8°; ●—at 45°.

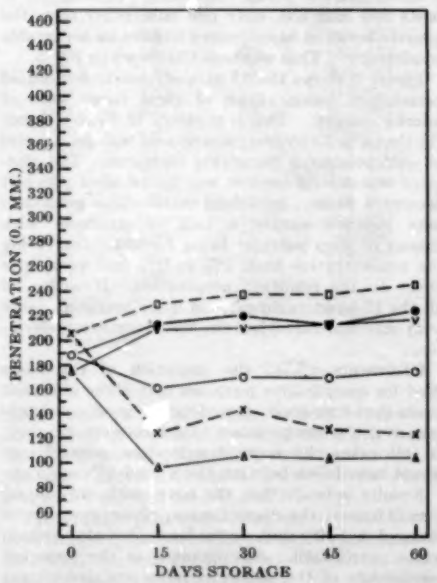

Fig. 4.—Consistency of ointment bases as determined by penetration measurements. Base 200: Δ—at 8°; v—at 45°. Base 225: ○—at 8°; ●—at 45°. Base 250: x—at 8°; □—at 45°.

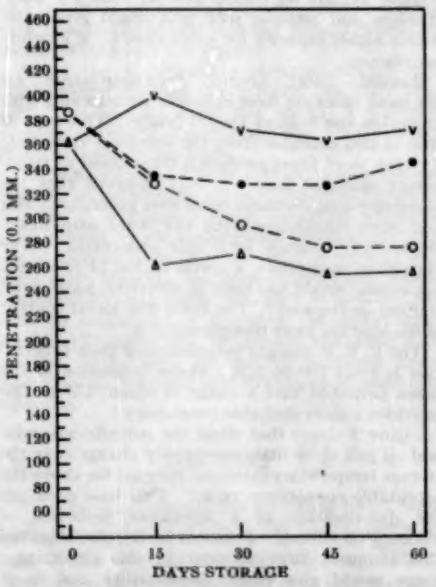

Fig. 6.—Consistency of ointment bases as determined by penetration measurements. Base 725: Δ—at 8°; v—at 45°. Base 730: ○—at 8°; ●—at 45°.

TABLE II.—STABILITY OF OINTMENTS STORED AT 45° FOR TWO MONTHS

Base	A Boric Acid 10%	B Ichthammol 10%	C Benzocaine 5%	D Iodine 4% KI 4% Glycerin 12%	E Ammoniated Mercury 5%	F Phenol 2% Glycerin 2%	G Benzoic Acid 6% Salicylic Acid 3%	H Coal Tar 3% Polysorbate 80 2%	I Sulfur 10%	J Zinc Oxide 10%	K Menthol 10% Methyl Salicylate 10%
75	SL(3), SM	SL(3), SM	SL(3), SM	SL(2)	SL(2), SM	NC	SL(3), SM	SL(3)	SL(2), SM	SL(2), SM	NC
100	NC	NC	NC	NC	NC	T-7	D	NC	NC	NC	NC
150	sl. S	NC	sl. S, D	NC	sl. S	T-7	NC	NC	sl. S	NC	NC
200	NC	NC	NC	sl. T-5	NC	T-3, T-7	NC	NC	NC	NC	NC
250	NC	NC	NC	B, T-6	NC	T-7	D	NC	NC	NC	NC
300	NC	NC	NC	B, T-6	NC	T-7	sl. St	NC	NC	NC	S
350	NC	NC	NC	B, T-6	NC	T-7	v. St.	NC	sl. D	NC	NC
450	NC	NC	D, S	B, T-6	D	T-7	sl. B, sl. S	NC	S	NC	NC
500	NC	sl. St	NC	B, S	NC	NC	S, T-5	NC	sl. D	sl. D	NC
550	NC	v. St.	NC	B, S	NC	NC	D, S, T-5	NC	D	D	NC
600	NC	NC	NC	sl. T-5	NC	NC	D, St, T-6	S	D	D	NC
650	NC	NC	NC	B, sl. T-5	NC	NC	D, S, T-5	NC	D	D	NC
700	NC	sl. T-5	sl. D	sl. T-5	NC	sl. T-5	T-5	NC	NC	sl. B	NC
750	D	T-5	D	B, T-5	B, sl. D, T-5	sl. T-5	T-5	NC	sl. B	B, T-6	T-6
800	sl. D, S, T-6	St	NC	SL(2), SM	sl. B, D	NC	D	NC	NC	NC	NC
850	SL(2), SM	St	sl. D	SL(2), SM	NC	NC	SL(2), SM	NC	D	D	NC

Key: Same as Table I.

Thus, the structure of these metallic soap-petrolatum ointment bases is very similar to the structure reported for polyethylene-mineral oil bases (2, 3). A large portion of both bases is liquid. The mobility of this liquid phase is believed responsible for the increased rate of drug release found in polyethylene-mineral oil bases (2, 3). This same increased rate of release could be expected from the liquid petrolatum-soap bases prepared in this study. Also, because of this high fluid content both the polyethylene and metallic soap gels would give bases with a higher capacity for solids than U. S. P. white petrolatum.

Results.—Short fibered, soap-petrolatum gel ointment bases are most desirable for softening with inunction and holding the oil firmly. This type of base is also desirable from the aesthetic viewpoint; for very short fibers produce a translucent or transparent ointment base. It was observed that the aluminum-soap ointment bases were generally clearer and more translucent than the other soap bases. Boner (11) concurs with this observation. The aluminum soaps have a metal cation of relatively low atomic weight and there is, therefore, a low metal content in the soap. The lower the metal content of the base the more transparent it is.

The U. S. P. accepts petrolatums if their penetration is from 100 to 275. Visual inspection of the bases indicated that a range of about 175 to 250 provided a more desirable consistency.

Figure 1 shows that while the polyethylene-mineral oil gels show little consistency change over the storage temperature extremes, they are far above the acceptable consistency range. This base possesses the disadvantage of a significant reduction of viscosity on milling. Foster, *et al.* (2), also observed this change. Any ointment in this penetration range would give easier spreadability and lower tube extrusion pressures than ointments in the conventional ranges.

Figure 1 also shows the marked consistency variations of two U. S. P. white petrolatums at the storage extremes. The average room temperature penetration line for U. S. P. white petrolatum was obtained from 21 penetration determinations on 11 different commercial samples.

Figures 2 and 3 show the slight consistency deviations and the acceptable penetration ranges of five mineral oil-metallic soap-gel ointment bases.

Only one series of metallic soap ointments showed moderately good consistencies throughout. Figure 4 shows the 200 series ointments. In addition to bases 200 and 250, only one base other than the quarter-hundred bases proved to have an acceptable consistency. That was base 850 shown on Fig. 5.

Figure 6 shows the 12-hydroxystearic acid-liquid petrolatum bases. Both of these bases were of inferior quality. This is contrary to Fiero's claims (1) that 2% 12-hydroxystearic acid will gel mineral oil and produce a liquefying ointment. The ointment was a solid until it was milled after which it became a grainy, semi-fluid mass. This graininess may indicate simply a lack of structure with masses of soap particles being formed. Increasing the concentration from 2% to 9% had very little effect on the resultant preparation. However, of all the 12-hydroxystearic acid bases prepared, those with only mineral oil had the least consistency deviation.

Comments.—With the exception of the bases used for comparative purposes only those ointment bases that had good or moderately good consistencies over the temperature extremes were graphed. In all cases the room temperature penetrations would have fallen between the 8° and 45° readings.

Results indicate that the bases with only liquid petrolatum as the discontinuous phase show better thermal stability than do the bases that also contain white petrolatum. Any changes in the apparent consistency of the ointment bases are due almost entirely to viscosity changes in the discontinuous phase (petrolatum-mineral-oil-lanolin phase) (4). Viscosity shows the expected inverse relationship to temperature change. Liquid petrolatum would

show the least viscosity change over the 8°–45° temperature range while white petrolatum or lanolin would exhibit considerably greater changes. Table III readily shows the quarter-hundred bases to be superior. The consistency differences between the storage temperature extremes are shown after sixty-days storage. Also, the difference after the bases, under varying storage conditions, were brought to 35° is given. Under "range" the extreme encountered during the sixty-day observations are given.

Almost all metallic soap gels studied exhibited thixotropy; that is, a decrease in consistency as a result of shearing, followed by an increase in consistency starting immediately after the shearing is stopped.

Results indicate that Aero aluminum stearate G–100 is the best of the metallic soaps employed, as regards formulation versatility. In addition, these

TABLE III.—CONSISTENCY SUMMARY AFTER SIXTY DAYS STORAGE

Base	Penetration Difference[a] 8°–45°	Penetration Difference[a] at 35°	Range[a]
60	51	34	298–373
75	327	69	123–450[b]
80	308	114	142–450[b]
100	250	35	109–359
125	68	3	185–253
150	320	31	130–450[b]
200	112	16	105–217
225	50	4	175–225
250	122	16	124–246
300	219	40	154–373
325	33	7	200–236
350	159	4	156–315
425	[c]	15	201–221
450	282	67	168–450[b]
500	281	65	150–431
525	39	7	205–244
550	289	87	112–401
600	237	79	157–394
625	20	14	183–211
650	211	40	154–365
700	278	56	156–434
725	114	19	256–370
730	69	26	275–356
750	208	54	149–357
800	237	75	148–385
825	29	22	218–262
850	109	32	116–225

[a] In penetration units (tenths of a millimeter).
[b] Maximum penetration.
[c] No 8° samples.

bases required a lower soap content to produce satisfactory gels than did any of the other bases. To give improved gel stability the manufacturer of this soap has replaced a small portion of the stearic acid with dimerized linoleic acid (5).

The addition of anhydrous lanolin to base 850 may have been responsible for its acceptable consistency. Evans (12) reports the use of wool fat alcohols as a peptizer (soap structure modifier) for calcium and other soap-base greases.

All of the half-hundred bases proved to be stable in the presence of water and capable of water absorption. Cholesterol, wool-wax alcohols, glyceryl monooleate, or other similar materials could also be added to impart water absorption.

Penetration results showed that more than half of the bases studied showed a gradual stiffening of the samples stored at 45° (when compared at 35° with the bases stored at 8° and room temperature). This elevated storage temperature may have been high enough for the soap fibers to regroup into a more random arrangement after being oriented by the milling operations.

Results of the ointment stability observations (Table II) show white petrolatum, U. S. P. to be an inferior base. The fact that petrolatum at elevated temperatures allows separation of incorporated solid medicaments constitutes its major disadvantage. Of the 165 ointments prepared from soap-gel bases only four (800D; 850A, D, and G) showed any separation of the medicaments.

It is apparent that each base and the ingredient to be incorporated into it is a separate problem. Few generalizations can be drawn. The 700 through 850 series ointments (12-hydroxystearic acid and sodium stearate bases) exhibited the most incompatibilities, and they would be generally the least desirable for over-all use.

In most instances where ointments are noted as too soft or too stiff this condition could be corrected by altering the soap concentration of the ointment base. This, together with proper selection of the ointment base would allow any of the tested ointments to be prepared without incompatibility.

The phenol ointments were usually too soft when they were initially prepared. As Table II shows some of these ointments became gummy after elevated temperature storage. Evans (12) has reported phenol to be an effective peptizer to produce extra long fibers in soap gels. This effect may have been rendered as a result of the elevated storage even though the phenol was not present when the initial gel structure was formed.

All of the K ointments (menthol-methyl salicylate ointment) were thin initially and after storage. This was no doubt due simply to physical thinning as a result of such a high fluid content being added to the ointment bases.

There would appear to be little danger in the use of many of the metallic soaps topically in regard to toxicity or irritation. Since aluminum stearates are approved for use in parenteral preparations they would necessarily be of a low order of toxicity and irritation. Mastitis ointments containing aluminum monostearate have been used in bovine teats with no indications of irritation. A report from Russia (13) states that fat greases thickened with alkaline earth soaps are suitable ointment bases since they do not cause skin irritations or other side reactions.

SUMMARY

Metallic soap-petrolatum ointment bases have been prepared and studied. It was found that gels of the metallic soaps with heavy liquid petrolatum produced the most desirable preparations. These ointment bases are superior to white petrolatum both in thermo-stability and elegance. Anhydrous lanolin added to these

newly developed bases provides water-absorption capacity with no incompatibility.

Of the eight metallic soaps studied Aero aluminum stearate G–100 proved to have the widest formulation application. It provided thermal stability to ointment bases containing white petrolatum and anhydrous lanolin in combination with heavy liquid petrolatum, as well as to the base mentioned above.

These new metallic soap gels proved to be superior or equal to other liquid petrolatum gels used as ointment bases.

Results varied in the 176 ointments prepared for compatibility observations. A vast majority of the ointments prepared from the metallic soap bases did not show the loss of homogeneity associated with the white petrolatum base ointments. Careful selection of the proper metallic soap-gel ointment bases would be required to successfully formulate compatible ointments.

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Studies on the Purity of Dimercaprol (BAL)*

By R. I. ELLIN, A. A. KONDRITZER, and D. H. ROSENBLATT

Variations in the toxicity of samples of dimercaprol have been related to the presence of a demonstrable chemical impurity. This impurity was present in the first distillates of fractionally distilled dimercaprol. Tests used to distinguish the presence of this impurity included: thiol sulfur titer, refractive index, lead acetate conductometric titrations, paper chromatography, and the formation of a solid derivative. The toxic impurity has been identified as 1,2,3-trimercaptopropane.

DIMERCAPROL [BRITISH ANTI-LEWISITE (BAL), 2,3-dimercapto-1-propanol], U. S. P. XV, was found to be an effective antidote for arsenical poisoning early in World War II and later was claimed to be of value in the treatment of several types of heavy metal poisoning (1). In the initial period of its commercial production and evaluation, variations in the toxicity of separate lots were noted (2). The preparation of dimercaprol from 2,3-dibromo-1-propanol and sodium trisulfide in the presence of hydrogen gave rise to difficultly separable impurities consisting mainly of 1,3-dimercapto-2-propanol and 1,2,3-trimercaptopropane (3). The process finally developed for the manufacture of dimercaprol was based on the bromination of allyl alcohol, followed by reaction of the 2,3-dibromo-1-propanol with sodium hydrosulfide (1). The application of this process by various laboratories has produced dimercaprol which varied widely in its toxicity to young adult white rats (4). The reason for this variable toxicity has not been reported.

Recent efforts to establish sources for the military procurement of dimercaprol created the need for a suitable specification to insure the absence of undesirable impurities. The purpose of this investigation was to determine the presence and nature of impurities in dimercaprol samples which are responsible for the observed variations in toxicity. This would make possible improvements in the synthesis, lead to satisfactory recovery of substandard lots of dimercaprol, and improve the chemical controls over its manufacture.

The observation that a small amount of yellow precipitate formed when dimercaprol was titrated with lead acetate in pyridine solution fur-

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ther indicated the presence of impurity. A number of lots of dimercaprol were screened by means of this test and a direct correlation was observed between turbidity formation and systemic toxicity of the dimercaprol sample.

The results of this report demonstrated the presence of 1,2,3-trimercaptopropane as an impurity in dimercaprol and indicated the desirability of a test to detect the presence of small quantities of this compound in dimercaprol and a method for removing it from dimercaprol samples.

EXPERIMENTAL

Samples from six lots of dimercaprol were examined. Lots *A* and *B* were of low toxicity and lots *C*, *D*, *E*, and *F* were of significantly higher toxicity. Samples, consisting of 100 to 200 ml., were fractionally distilled through a 45-cm. Vigreux column under a stream of oxygen-free nitrogen to provide four distillate fractions. The fractions represented 25, 30, 30, and 10% of the initial volume; a 5% residue remained. They are referred to as fractions 1–5. The distillation pressures were kept in the 0.3 to 1.0 mm. range and the distillation temperature varied from about 82° initially to 92°. Except for fraction 1, the distillation temperatures of the various fractions were very close to one another. Fraction 1 from samples *A* and *B* were higher boiling by 5 to 6° than similar fractions of *C–F*. Although not all fractions were tested for toxicity, sample *C* and fractions *C*-1 and *C*-2 were found to be definitely more toxic than sample *A* or fraction *A*-1 (4).

Isolation of Dimercaprol Impurity.—Fractions 1–4 of samples *A–F* were tested for water solubility by adding approximately 35 volumes of water for each volume of sample. As one part of BAL is soluble in about 15 parts of water, all the BAL should have dissolved; however, two layers were noted. Upon centrifuging and decanting the supernatant liquid, the insoluble product was isolated. It was purified by solution in methanol, reprecipitation with water, isolation as above, and finally dried in a vacuum desiccator over phosphorous pentoxide.

Identification of Dimercaprol Impurity

The isolated impurity was identified by the following tests:

Titration for Thiol Sulfur.—Approximately 100 mg. of sample was weighed in a glass weighing cup which was placed into a 150-ml. iodine flask. About 30 ml. of methanol was then added and the flask swirled to dissolve the sample. The solution was titrated with 0.1 *N* iodine solution until addition of one drop produced a faint yellow color.

Lead Acetate Turbidity Test.—The observation was made that the derivative formed by reacting BAL with lead acetate under appropriate solvent conditions was considerably more soluble than derivatives of possible thiolic impurities. This finding was made the basis of a nonspecific, semiquantitative test for impurities in BAL samples, since solutions of BAL containing such impurities would become appreciably more turbid on addition of lead acetate than BAL samples containing little or no impurity.

Lead Acetate Reagent.—Weigh out exactly 0.417 (±0.001) gram of lead acetate (neutral), i. e., $Pb(CH_3COO)_2 \cdot 3H_2O$, and transfer to a 100-ml. volumetric flask. Add 2 ml. of glacial acetic acid and shake until the lead acetate is dissolved. Make up to volume with C. P. pyridine.

Test Solution.—Into a 100-ml. flask weigh at least 124 mg. of BAL sample to be tested and dilute to volume with N,N-dimethylformamide. If the weight of BAL taken is greater than 124 mg., adjust the concentration to 124 mg. per cent by diluting with the appropriate amount of N,N-dimethylformamide.

Procedure.—To 5 ml. of the test solution in a 10-ml. test tube add 5 ml. of lead acetate reagent. Begin timing, mix the solutions thoroughly, and then transfer to a 19-mm. Coleman round cuvette. At the end of exactly ten minutes, read at 425 millimicrons on a Coleman Junior spectrophotometer (against a blank composed of 5 ml. lead acetate reagent plus 5 ml. N,N-dimethylformamide).

Absorbance values of each sample and corresponding fractions are given in Table I. Samples of low toxicity BAL had absorbance readings of 0.200 and lower; high toxicity BAL samples had absorbance readings of 0.600 and higher.

Refractive Index.—Refractive indexes of all samples were measured with a Bausch and Lomb Abbé "56" Refractometer.

Results of the above three tests are given in Table I.

TABLE I.—ANALYSIS OF VARIOUS LOTS AND FRACTIONS OF MERCAPTANS

Lot	Fraction	Thiol Sulfur, %	Lead Acetate Turbidity Reading (Absorbance)	Refractive Index $n_D^{25.0}$
A		51.16	0.150	1.5709
	1	51.97	0.169	1.5709
	2	52.00	0.113	1.5703
	3	51.94	0.128	1.5698
B		50.93	0.144	1.5709
	1	51.35	0.196	1.5708
	2	51.45	0.120	1.5701
	3	51.70	0.107	1.5688
C		51.48	0.790	1.5713
	1	53.44	1.40	1.5722
	2	52.42	0.530	1.5703
	3	52.23	0.120	1.5700
D		51.24	1.10	1.5731
	1	53.07	1.25	1.5743
	2	52.03	0.950	1.5723
	3	51.36	0.157	1.5709
E		50.22	0.820	1.5739
	1	53.90	1.50	1.5749
	2	53.17	0.950	1.5709
	3	51.92	0.152	1.5700
F		50.74	1.30	1.5747
	1	54.67	1.70	1.5758
	2	52.54	1.05	1.5720
	3	52.19	0.180	1.5708
Trimercaptopropane		68.35 (found) 68.57 (theory)	Too high to read	1.6078
Isolated impurities		64.01–67.31	Too high to read	1.5851–1.6011

Conductometric Titrations.—Lead acetate, 0.1 *M*, in pyridine was used as the titrant. Titrations were carried out in pyridine on approximately 0.05 *M* solutions each of sample *A*, the impurity isolated from distillate fraction *C*-1, and 1,2,3-trimercaptopropane. The operation and procedure have been described (5). The results are plotted in Fig. 1, which shows that the conductometric curve for the isolated impurity clearly resembles that obtained using 1,2,3-trimercaptopropane.

Filter Paper Chromatography.—Six 15-inch strips of Whatman No. 1 paper, $1^1/_2$ inches wide, were marked, impregnated with 1-microliter samples of lots *A*–*F*, and placed in a large cylindrical glass jar arranged for ascending chromatography. A mixture of equal volumes of benzene, heptane, methanol, and water was placed in a dish on the bottom of the chamber. After standing overnight to attain equilibrium, the strips were lowered into the upper organic phase. After four to five hours, the solvent front was marked, the strips removed from the chamber, and dried. On spraying the papers with a platinic potassium iodide reagent, made by adding 2 ml. of a 10% platinic chloride solution to 1 gram of potassium iodide in 98 ml. of water, all chromatograms showed two yellow bands, one having an R_f value of 0.65 and the other an R_f value of 0.93. Repetition with 1 microliter of the isolated impurity produced the same results, while similar chromatograms with 1,2,3-trimercaptopropane showed only one band that had an R_f value of 0.92.

Preparation of the Tris-2,4-dinitrophenyl Derivative.—The 1,2,3-tris-(2,4-dinitrophenylmercapto) propane derivative was prepared, using the method of Grogan, *et al.* (6), by dissolving 0.005 mole of trithiol and 0.015 mole of 2,4-dinitrochlorobenzene in 20 ml. of ethanol and titrating with a 0.1 *M* alcoholic solution of KOH. The amorphous product was recrystallized from butanone, m. p. 201–203° (uncorr.). The 2,4-dinitrophenyl derivative of the isolated impurity, prepared similarly, had the same melting point and gave no melting point depression when mixed with authentic 1,2,3-tris-(2,4-dinitrophenylmercapto) propane.

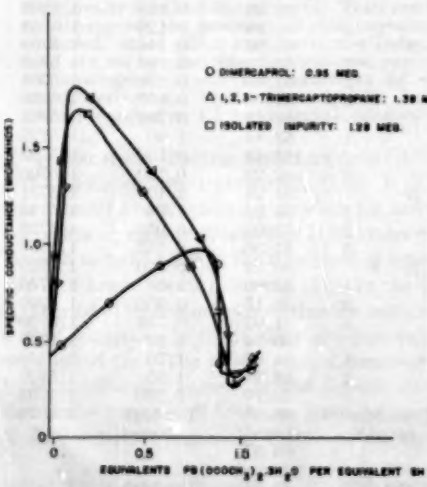

Fig. 1.—Conductance of mercaptans in pyridine on addition of 0.100 *M* $Pb(OCOCH_3)_2 \cdot 3H_2O$

The 1,2,3-trimercaptopropane used in the above tests and reactions was synthesized from tris-acetylmercaptopropane according to the method of Miles and Owen (7).

DISCUSSION

The chemical and physical data indicate that the impurity which is present in all the batches of dimercaprol tested and which was isolated from batches having high toxicity is 1,2,3-trimercaptopropane. This impurity could arise during the manufacturing process via the following sequence of reactions:

Primary reaction—

$$CH_2{=}CHCH_2OH + Br_2 \rightarrow CH_2BrCHBrCH_2OH$$

(2,3-dibromo-1-propanol, b. p. 219°C.)

$$\xrightarrow{NaSH} CH_2SHCH_2SHCH_2OH$$

(dimercaprol, b. p. 85–90°/1 mm.)

Other possible reactions—

$$CH_2{=}CHCH_2Br + Br_2 \longrightarrow CH_2Br\,CHBr\,CH_2Br$$

$$CH_2{=}CHCH_2OH + Br_2 + H_2O \nearrow$$

(1,2,3-tribromopropane, b. p. 220°)

$$\xrightarrow{NaSH} CH_2SHCHSHCH_2SH$$

(1,2,3-trimercaptopropane, b. p. 80°/1.5 mm.)

The presence of a small quantity of allyl halide as an impurity in the allyl alcohol could account for the formation of tribromopropane, which would distill together with dibromopropanol since both compounds have almost identical boiling points. Another possibility is the presence of moisture during the bromination step which could result in the formation of small quantities of tribromopropane. During the thionation procedure whereby the dibromopropanol is converted to dimercaprol, the tribromopropane would be converted to trimercaptopropane.

The greater toxicity of 1,2,3-trimercaptopropane when compared to that of dimercaprol on parenteral injection into rats and rabbits has been reported (8). Investigations are now in progress to prevent or reduce to a minimum the formation of the trithiol impurity during the manufacturing process, and to eliminate the impurity whenever it appears in the dimercaprol product. It is anticipated that as a result of these studies an improved specification and better methods for characterizing dimercaprol will be forthcoming.

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A Rapid Determination of the Relative Purity of Vitamin B_{12} (Cyanocobalamin) in Pharmaceutical Products

By C. F. BRUENING, W. L. HALL, and O. L. KLINE*

A procedure is described involving the determination of absorptivities at 341 and 376 mμ and their ratio. Solutions of crystalline cyanocobalamin gave maximum absorptivities at 341 and 376 mμ of 80.4 and 80.9, respectively. The average ratio was 0.990 with none exceeding 0.998. A number of pharmaceutical preparations of cyanocobalamin injection gave ratios not in excess of 1.02. Several commercial preparations known to contain impure cyanocobalamin gave ratios up to 1.24. Solutions containing cobalamin concentrate N. F. and potential impurities of cyanocobalamin injection gave ratios ranging as high as 1.48. Validity of the procedure was established by comparison with reference cyanocobalamin solutions, and with a "Purity Index" obtained from the results of the Radioisotope Tracer Method for Cobalamins and the U. S. P. Spectrophotometric Method for Cyanocobalamin.

IN THE QUANTITATIVE DETERMINATION of highly purified solutions of cyanocobalamin the spectrophotometric method described in U. S. Pharmacopeia XV (1) has been widely used. This involves measurement of radiant energy absorption at 361 mμ. When it became evident that this method did not differentiate between the biologically active cobalamin compounds and the structurally similar but inactive red pigments, a radioisotope tracer method was devised (2). This method has been adopted by both the U. S. Pharmacopeia (3) and the National Formulary (4) and in the latter for application to cobalamin concentrate N. F. (5).

Application in this laboratory of these two methods to an extensive series of pharmaceutical products offered as vitamin B_{12} injections U. S. P., has provided an estimate of purity of the vitamin used in the preparations tested. Arbitrarily, we have expressed this relationship as "Purity Index," calculated by dividing the amount of cyanocobalamin found by the tracer method by the amount found by the spectrophotometric method. This has been a useful means in determining whether or not the products examined met the U. S. P. specifications.

In a spectrophotometric study of this series it was observed that the radiant energy absorption at the shorter wavelengths was abnormally high for those products known to contain impurities. To use this observation in a quantitative manner the absorption ratio at 341 and 376 mμ was compared for solutions of pure cyanocobalamin and for the impure vitamin preparations. This was found to provide a rapid spectrophotometric method for determining relative purity and to serve as a preliminary sorting procedure for control purposes.

Briefly, the determination involves the measurement of radiant energy absorption at three different wavelengths, 341, 361, and 376 mμ. The ratio of the absorption at 341 and 376 mμ, together with quantitative absorption values at these two wavelengths, gives an index of the purity of the cyanocobalamin. The validity of the method was established by using a solution of crystalline cyanocobalamin as a reference standard, or by comparing results with the Purity Index found for each sample by use of a combination of the tracer and the U. S. P. spectrophotometric methods. It has been shown to be reliable for commercial preparations such as cyanocobalamin U. S. P. XV, cyanocobalamin injection U. S. P. XV, and injections of cobalamin concentrate solution. It is applicable to solutions containing 30 or more mcg./ml. of cyanocobalamin. It is rapid, and requires no special apparatus such as the radioactivity counting assembly of the tracer method.

EXPERIMENTAL

The proposed determination originated in the observation that a number of products containing impure cyanocobalamin gave abnormally high absorption at about 340 mμ. To investigate the impurity absorption spectrum over a definite wavelength range, a standard reference solution of cyanocobalamin was prepared which, according to the value by the tracer method, had the same concentration of cyanocobalamin as the sample under examination. A differential spectrophotometric analysis was then made for each sample using a recording spectrophotometer (Beckman Model DK-1) between 320 and 600 mμ wavelengths. In this procedure the standard solution was placed in the reference beam of the instrument and the sample solution in the sample beam. Then at any particular wavelength, since both standard and sample solutions

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contain identical amounts of cyanocobalamin, the reference solution "balances out" or "optically neutralizes" the absorption of cyanocobalamin in the sample solution. With pure solutions a curve of zero absorption was obtained, appearing as a horizontal line on the chart. When impurities were present, however, the result was an absorption curve deviating from the horizontal line. In all samples it was observed that the absorption curve was a horizontal line as the wavelength decreased from 600 mμ to approximately 375 mμ where the line assumed a slight slope which continued to approximately 340 mμ and became approximately horizontal again. The curves showed that in all cases the absorbance of the sample at approximately 340 mμ was higher than that at approximately 375 mμ. In most of the samples examined the curve between 340 and 375 mμ was approximately linear, but in a few it was nonlinear. These observations suggested that the cyanocobalamin solutions contained impurities that absorb strongly in the region of 340 mμ, with little or no abnormal absorption at approximately 376 mμ. Nearly all of the samples showed an approximate proportional relationship between increased absorption at 340 mμ and amount of impurities.

To provide an index of relative purity of cyanocobalamin, wavelengths of 341 and 376 mμ were selected for absorption measurements. At these wavelengths cyanocobalamin gives a ratio of absorptivities of approximately 1.0, and a deviation from this indicates the absorption effect of impurities at lower wavelengths.

Essentially, this ratio is a simplified form of an "Impurity Index" (6) used in many industrial organic analyses to measure quantitatively the amount of unknown absorbing impurities present in a sample being processed. In this report this ratio is used to denote the relative purity; the absolute purity has not been evaluated because it is believed that the commercial samples examined here, especially those of high impurity content, have been manufactured under a variety of conditions and the background impurity absorption curves are not sufficiently uniform to permit accurate estimate of the amount of impurities. In those cases where the absolute purity is needed, the use of the tracer method is indicated. It is emphasized that only where impurities are low or nonexistent is the U. S. P. spectrophotometric method, which measures the absorbance at 361 mμ, satisfactory for quantitative purposes.

In a few of the sample solutions containing impure cyanocobalamin it was observed that the ratio was only slightly in excess of that for cyanocobalamin, but absorption at both 341 and 376 mμ was abnormally high. To detect this type of deviation, the absorptivities at 341 and 376 mμ were calculated from absorbance measurements at 361 mμ and each compared to the corresponding one for cyanocobalamin.

DETERMINATION

Dissolve the solid cyanocobalamin sample in sufficient water or dilute cyanocobalamin injections with sufficient water to give a final concentration of cyanocobalamin of approximately 40 mcg./ml.

Measure carefully the absorbance of the sample solution at 341, 361, and 376 mμ with a Beckman Model DU spectrophotometer, using the tungsten lamp light source and corex filter. Use 1-cm., quartz, matched cells with water as the reference solvent. Calculate the ratio of the absorption at 341 and 376 mμ, using either the absorbance or absorptivity values. Also calculate the absorptivity values at 341 and 376 mμ assuming that the absorptivity at 361 mμ is 207.

It may be of value to include in the determination, measurement of a reference standard solution of cyanocobalamin of high purity. Initially the use of such standard would establish absorbance and ratio values for the spectrophotometer employed. Its use would also improve precision of the determination by minimizing errors from improper setting of the wavelength scale or faulty performance of the instrument.

For this purpose prepare a solution of the reference cyanocobalamin that contains approximately 40 mcg./ml. and make the required absorbance readings at each wavelength, using the undisturbed wavelength setting of the sample measurement. Such reference measurements would be a basis for correction, where necessary.

DISCUSSION OF RESULTS

The absorptivity and ratio values for crystalline cyanocobalamin are shown in Table I. The term "absorptivity," as used here, is defined in the U. S. Pharmacopeia (1) as "The absorbance of a solution containing 1 Gm. per 100 ml. contained in a cell having an absorption path of 1 cm. Symbol: *a*." Here and throughout this report, in order to avoid the necessity of weighing out the crystalline sample or determining the weight of the cyanocobalamin of injections, an absorbance reading at 361 mμ was made as required by the procedure. The absorptivity at this wavelength for cyanocobalamin was assumed to be 207 (1). From the relative absorbance values at the three wavelengths used in the procedure, and with the absorptivity at 361 mμ assumed to be 207, the absorptivity at 341 and 376 mμ is readily calculated.

The four different samples of cyanocobalamin used were manufactured at different times during the period 1950 to 1955. The purity of cyanocobalamin samples *A* and *B* were demonstrated by the use of a countercurrent procedure; this type of analysis is particularly suitable for purity determinations of cyanocobalamin. By use of the procedure "Countercurrent Analysis of Vitamin B_{12}" (7), Sample *A* had a purity of 97.0% and Sample *B* 98.5%. High spectrophotometric purity of each sample has been demonstrated frequently since both have served as spectrophotometric reference standards in our laboratory. Sample *B* was a portion of cyanocobalamin that was used in preparing the U. S. P. Reference Standard. Both samples have been used in our laboratory also as reference standards in the tracer method, and thus all values for Purity Index in this report are related to either Sample *A* or Sample *B*. Samples *C* and *D* were commercially available cyanocobalamin samples and were used for comparison purposes with Samples *A* and *B*.

Water solutions were prepared to serve as references for solid cyanocobalamin samples, while water solutions containing 1.5% benzyl alcohol by

TABLE I.—ABSORPTIVITY OF CRYSTALLINE CYANOCOBALAMIN

Sample	Solvent	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, a_{341} mμ/ a_{376} mμ
A	Water	80.3	80.7	0.995
A	1.5% Benzyl alcohol	80.4	80.6	0.998
B	Water	79.3	80.3	0.988
B	1.5% Benzyl alcohol	79.4	80.3	0.989
C	Water	79.0	80.3	0.984
C	1.5% Benzyl alcohol	79.4	80.9	0.981
D	Water	79.4	80.3	0.989
D	1.5% Benzyl alcohol	80.0	80.7	0.991
Av.	Water	79.5	80.4	0.989
Av.	1.5% Benzyl alcohol	79.8	80.6	0.990

volume were prepared for comparison with cyanocobalamin injections. A typical cyanocobalamin injection contains isotonic sodium chloride and 1.5% by volume benzyl alcohol. It was observed that isotonic sodium chloride gave no absorption at any of the three wavelengths involved in the determination. However, benzyl alcohol does absorb to some extent, and it was necessary to include benzyl alcohol in the reference solution for injections. The actual average increase in absorption due to the addition of benzyl alcohol, as noted in Table I, was very small. From the data in Table I a given sample of cyanocobalamin dissolved in water, or water containing 1.5% benzyl alcohol, should not give an absorptivity in excess of 80.4 at 341 mμ or 80.9 at 376 mμ. The ratio of absorptivity at 341 mμ and 376 mμ should not exceed 0.998. The presence of typical absorbing impurities will tend to increase both the absorptivity at 341 mμ and the ratio; in some cases, the absorptivity at 376 mμ may also increase.

Table II shows the absorptivity and ratios of samples of cyanocobalamin injections having a purity index of less than 95%. The history of these samples indicated that they were made from some type of oral grade solids, possibly with additional purification. The deviation from U. S. P. standards for injections of crystalline cyanocobalamin was corroborated by the fact that all of these samples contained unaccountable excess solids. A final proof that at least three of these samples contained impurities that absorb abnormally high at 341 mμ was shown by the fact that, after they had been examined by the tracer method (which yields a solution of cyanocobalamin in pure form), the ratios on the final solutions were essentially those for cyanocobalamin. Sample 1, with a ratio initially of 1.086, dropped to 1.000; Sample 4 changed from 1.085 to 0.983; and Sample 5 from 1.242 to 1.025. By the same procedure, it has been demonstrated that samples containing crystalline cyanocobalamin give initial and final ratios that are essentially identical.

The data of Table II show that where impure cyanocobalamin samples having an indicated purity of less than 91.3% (Samples 1 to 4), a ratio of 1.085 or more is obtained as contrasted to the reference ratio of 0.990 for cyanocobalamin solutions (Table I). Sample 5, with an indicated purity of 94.2%, showed an unusually high ratio of 1.242. This sample was of special interest because the impurity absorption curve (resembling a sine curve) was quite different from the other samples of impure cyanocobalamin injections shown in Table II. Between wavelengths 341 and 376 mμ the absorption curve was nonlinear, with negligible absorption at 361 mμ and 376 mμ, but with considerable absorption at 350 mμ and 341 mμ. Samples 6 and 7, with purity indexes of 93.8% and 94.8%, showed ratios of 1.021 and 1.012, respectively. These ratios do not exceed that for cyanocobalamin by too large amounts, but they would be viewed with suspicion because the absorptivities at 341 and 376 mμ exceed those for cyanocobalamin solutions by considerable amounts.

TABLE II.—ABSORPTIVITY OF PHARMACEUTICAL PREPARATIONS OF CYANOCOBALAMIN INJECTION U. S. P. HAVING A PURITY INDEX LESS THAN 95%

Sample	Cyanocobalamin Concn., mcg./ml.	Purity Index,* %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, a_{341} mμ/ a_{376} mμ
1	1,000	89.6	86.8	79.9	1.086
2	1,000	90.2	94.1	86.3	1.090
3	1,000	90.8	96.8	86.5	1.122
4	1,000	91.3	86.5	79.7	1.085
5	1,000	94.2	101.5	81.7	1.242
6	1,000	93.8	83.8	82.1	1.021
7	1,000	94.8	84.5	83.5	1.012

* Purity Index is obtained by dividing the cyanocobalamin content determined by the Radioisotope Tracer Method by the cyanocobalamin content determined by the U. S. P. Spectrophotometric Method.

Sample 7 has an especially high absorptivity at 376 mμ indicating a background impurity absorption curve somewhat horizontal, but of high magnitude. Samples 6 and 7 no doubt contain purified oral grade solids, and if encountered in our laboratory on a routine basis, would indicate the use of the tracer method and a solids determination to indicate compliance with the U. S. P. specification for cyanocobalmin injection.

Table III shows the absorptivities and ratios of pharmaceutical preparations found to have a purity index of more than 95%. The quality of the cyanocobalamin used in these samples is unknown. Arbitrarily, a purity index in excess of 95% was chosen to serve as an indication of the presence of crystalline vitamin B_{12} (cyanocobalamin) where the product was so labeled. The 95% criterion was chosen because of the U. S. Pharmacopeia requirement (1) that cyanocobalamin "has a purity of not less than 95% on the dried basis." It should be noted, however, that the two criteria are not identical as illustrated by the following hypothetical cases. One sample, containing 95 parts of anhydrous pure cyanocobalamin and 5 parts of sodium chloride, would assay 95% by the U. S. P. method; since that method compares absorbance at 361 mμ to dry weight of sample and would assay 100% Purity Index as this method compares cyanocobalamin alone to absorbance at 361 mμ. Another sample, containing 95 parts of anhydrous pure cyanocobalamin and 5 parts of "red pigments" (such as Sample 6 of Table IV), would assay 100% by the

TABLE III.—ABSORPTIVITY OF PHARMACEUTICAL PREPARATIONS OF CYANOCOBALAMIN INJECTION U. S. P. HAVING A PURITY INDEX MORE THAN 95%

Sample	Cyanocobalamin Concn. mcg./ml.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
1	2,000	101.5	81.4	81.8	0.995
2	1,000	100.5	81.1	82.3	0.985
3	30	100.3	81.8	81.5	1.004
4	1,000	100.2	80.1	79.4	1.009
5	30	98.9	82.0	81.4	1.007
6	1,000	98.9	82.3	82.3	1.000
7	1,000	98.8	81.1	81.2	0.999
8	100	98.8	79.9	79.4	1.006
9	1,000	98.7	82.2	81.8	1.006
10	50	98.7	80.1	80.1	1.000
11	1,000	98.6	83.0	81.6	1.017
12	100	98.5	81.8	81.2	1.007
13	2,000	97.4	79.0	79.9	0.989
14	100	96.5	82.5	81.7	1.010
15	1,000	96.3	80.9	81.2	0.996
16	1,000	95.5	84.0	81.8	1.027
17	2,000	95.5	79.7	81.5	0.978

[a] See footnote—Table II.

oral grade, defined as Cobalamin Concentrate N. F. Sample 2 contained, in addition, 200 mcg./Gm. of a specially prepared red pigment. The relationship between purity index and high ratio is apparent in these two samples. Sample 3 was a cobalamin concentrate which gave a high ratio and high absorptivities at both 341 and 376 mμ. Sample 4 was a high concentration vitamin B_{12} soluble solids sample which, according to the purity index and the ratio, contained a relatively large amount of impurities. Sample 5 was pseudo vitamin B_{12}, isolated from an anaerobic fermentation process in crystalline form and reported present in a commercial vitamin B_{12} feed supplement (8). Sample 6 was a sample of "red pigments" which occurred in the waste stream of a commercial process for preparing cyanocobalamin. The "red pigments" accompany cobalamins rather closely through certain manufacturing steps and are separated only with difficulty, usually on a chromatographic column (2). They are present, apparently, in all cobalamin concentrates N. F., but are removed from cyanocobalamin U. S. P. As poor manufacturing or incomplete processing may not remove these "red pigments" from cyanocobalamin to be used in injections, it is important that any proposed method detect this type of contamination. It is believed the proposed determination which gives a ratio of 1.478 with accompanying high absorptivities at 341 and 376 mμ is sufficiently sensitive to detect small amounts of these "red pigments" in cyanocobalamin preparations. Thus, on a theoretical basis, using this sample as a typical pigment, a synthetic mixture of 95% cyanocobalamin and 5% of "red pigments" would give (assuming Beer's law applies) a ratio of about 1.015. In contrast, the tracer method would give a purity index of 95.2%.

TABLE IV.—ABSORPTIVITY OF COBALAMIN CONCENTRATE PREPARATIONS AND POTENTIAL IMPURITIES OF CYANOCOBALAMIN

Sample	Vitamin B_{12} Activity, mcg./Gm.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, $a_{341\ m\mu}/a_{376\ m\mu}$
1. Collaborative Sample 1	1,076	75.6	108.9	88.5	1.231
2. Collaborative Sample 2	1,067	67.6	106.2	88.3	1.203
3. Cobalamin Conc. N. F. X	500	93.0	90.6	86.9	1.043
4. Vitamin B_{12} Soluble Solids	34,000	91.6	99.3	90.0	1.103
5. Crystalline Pseudo Vitamin B_{12}	..	1	87.4	63.1	1.385
6. "Red Pigments"	..	4	156.7	106.0	1.478
7. Crystalline Red Pigment	..	0	80.2	82.1	0.977

[a] See footnote—Table II.

U. S. P. method and 95% by the purity index method.

Inspection of Table III shows that with the exception of Sample 16, all samples have a maximum ratio of 1.017 and maximum absorptivities of 83.0 and 82.3 at 341 and 376 mμ, respectively. With this exception, it would seem logical to select for sorting purposes the following maximum values for injections made from cyanocobalamin, U. S. P.: Ratio $a_{341\ m\mu}/a_{376\ m\mu} = 1.020$; Absorptivity at 341 mμ = 83.0; Absorptivity at 376 mμ = 82.5. Inspection of the ratio values of Table III shows that 15 out of 17 samples do not exceed the reference ratio for cyanocobalamin (0.990) by more than 2% (i. e., not in excess of 1.010). It appears that in view of the conformity of these samples with the reference samples, the ratio selection of a maximum of 1.020 is justified in a rapid determination for sorting purposes.

The absorptivities and ratios of cobalamin concentrate preparations and some potential impurities of cyanocobalamin are shown in Table IV. In all samples, aqueous solutions were prepared for measurements by removing insoluble material with filtering or centrifuging. Samples 1 and 2 were used in the collaborative study "Cobalamin Assay by the Radioisotope Tracer Method" (2), and contained commercially prepared vitamin B_{12} solids of

Sample 7 of Table IV was a red crystalline substance isolated from the "red pigments" fraction of a commercial vitamin B_{12} fermentation process. It is considered a definite chemical compound, while "red pigments" represented by sample 6 may contain several similar compounds. This red pigment was used in the collaborative study mentioned above, having been added to sample 2 of Table IV. It was used because its ultraviolet absorption spectrum in water was somewhat similar to that of cyanocobalamin, so that spectral differentiation was difficult. In the present studies its absorptivities and ratios were essentially the same as for cyanocobalamin; thus it cannot be differentiated by this means. On studying this pigment further, by a dif-

ferential spectrophotometric analysis using cyanocobalamin as the reference sample, a straight horizontal line was obtained on the absorbance curve, indicating, within experimental error, that this red pigment and cyanocobalamin have identical absorption curves between 600 and 320 mμ. It is interesting to note that this sample which showed no biological activity did not interfere in the tracer determination of sample 2 in the aforementioned collaborative study. From a practical viewpoint, the inapplicability of the proposed determination is not too serious because it is unlikely that cyanocobalamin will be contaminated with only one such specially purified fraction from "red pigments."

Table V shows the absorptivity and ratio of seven commercial samples of injections made from cobalamin concentrate solution, preparations now available for clinical use. They appear to contain cyanocobalamin of higher purity than that in cobalamin concentrate N. F., but not as pure as cyanocobalamin U. S. P. The results in Table V show that all samples gave a purity index of 94.8% or more. With the exception of Sample 1, the ratio range was between 0.992 and 1.023. Thus most commercial samples can be expected to give ratios below 1.025, which is slightly higher than the maximum ratio of 1.020 expected from injections made from cyanocobalamin. It is interesting to note that Sample 7, with an indicated purity of 99.4%, gave an average ratio of 0.994 which is very close to the cyanocobalamin reference ratio of 0.990.

Because of the fact that the ratio of the absorptivity at 341 and 376 mμ is a rather sensitive index for determining relative purity of cyanocobalamin in various preparations, a brief study was made of reproducibility of results. Table V lists, for each sample, results of two separate determinations. It can be seen that maximum deviation in the ratio was for Samples 1 and 2, approximately 1.3%. The average difference between duplicates of all the samples was 0.6%.

This degree of reproducibility is not considered excessive when the following experimental facts are considered: *first*, the duplicate determinations in Table V and all other determinations in this report were made in essentially the same manner as routine analyses in a typical control laboratory. Four clean, matched silica cells were used interchangeably for all determinations in this report. *Second*, the reproducibility and accuracy of the method is probably largely dependent on the performance of the spectrophotometer. In a typical analysis where the concentration of the solution is about 40 mcg./ml. cyanocobalamin, the absorbance reading at 341 mμ and 376 mμ is about 0.325, and at 361 mμ about 0.82. The slit widths used (sensitivity control of midpoint) at 341, 361, and 376 mμ were approximately 0.15, 0.095, and 0.08 mm., respectively. A frequently occurring reproducibility of a particular Beckman Model DU may, in individual samples being measured for absorbance at the three wavelengths, be of the magnitude of about 0.003 absorbance unit. If 0.003 error is made in both the 341 and 376 mμ readings, and in opposite directions, it is apparent that the calculated ratio of a typical sample might contain an error of 1.8%. Thus, the reproducibility of about 1.3% with samples 1 and 2 appears to be reasonable and probably arises from the performance of the Beckman rather than defects

TABLE V.—ABSORPTIVITY OF COMMERCIAL INJECTIONS MADE FROM COBALAMIN CONCENTRATE SOLUTIONS

Sample	Cyanocobalamin Concn. mcg./ml.	Purity Index,[a] %	Absorptivity 341 mμ	Absorptivity 376 mμ	Ratio, a_{341} mμ/ a_{376} mμ
1	1,000	96.5	87.3	81.3	1.074
	1,000	96.5	87.2	82.3	1.060
2	1,000	95.3	81.4	80.5	1.011
	1,000	95.3	81.0	81.2	0.998
3	1,000	96.4	82.8	80.9	1.023
	1,000	96.4	83.1	81.8	1.016
4	100	94.8	83.6	82.6	1.012
	100	94.8	84.0	83.1	1.011
5	1,000	96.1	81.7	81.9	0.998
	1,000	96.1	82.0	82.0	1.000
6	1,000	96.9	82.5	82.0	1.006
	1,000	96.9	83.1	82.5	1.007
7	1,000	99.4	82.0	82.7	0.992
	1,000	99.4	82.3	82.6	0.996

[a] See footnote—Table II.

in the method. A word of caution, however, should be considered in making absorbance readings on the Beckman Model DU in this determination. The readings at 341 and 376 mμ are taken on a rather steep portion of the curve where the absorbance is changing rapidly with wavelength, making it imperative that the wavelength setting be made with care. In order to obtain the best precision, the following procedure is recommended: in addition to making an absorbance reading at 341 or 376 mμ on a sample, after the wavelength dial has been carefully adjusted, also make a reading on a reference sample of cyanocobalamin and compare the difference in ratios. This actual difference will mean much more than individual absolute values because it will be compensated not only for an improper setting of the wavelength dial, but also for small permissive errors in the wavelength scale of the instrument. The absorbance reading at 361 mμ caused no difficulty and it is doubted if the reproducibility of most samples in this report exceeded 0.5%. *Third*, since the validity of the proposed determination is based on comparison with the purity index procedure, it was considered desirable to determine the reproducibility of the latter, which is dependent largely upon the variation of the tracer method with little variability being contributed by the U. S. P. spectrophotometric method. To determine the variability of the indicated purity procedure, duplicate determinations were made on five of the samples under study. Sample 3 of Table II gave a difference between duplicates of 1.1; Sample 5 of Table II, 1.0; Sample 6 of Table II, 1.4; Sample 16 of Table III, 0.4; and Sample 1 of Table V, 1.1. On a percentage basis, the average reproducibility was 1.1; and the maximum, 1.5. Thus, the purity index or the ratio of absorptivity at 341 and 376 mμ have approximately the same degree of reproducibility.

SUMMARY

A rapid reproducible spectrophotometric procedure is described for determining the relative purity of cyanocobalamin that is applicable to commercial preparations. It involves measuring

the absorptivity values at 341 mμ and 376 mμ, and calculation of the ratio of the absorptivities at these two wavelengths. The procedure can be used on solutions containing 30 or more mcg./ml. of cyanocobalamin. The validity of the procedure was affirmed by comparison with solutions of cyanocobalamin of known purity, or a purity index calculated from the results of the Radioisotope Tracer and the U. S. P. Spectrophotometric Methods. The procedure was used on a variety of samples and the relative purity of cyanocobalamin estimated was generally in agreement with that given by the purity index. The procedure serves as a rapid sorting device, but until broader experience is available, final reliance must be placed in the use of the purity index involving the Radioisotope Tracer Method.

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The Metabolic Fate of a Hexamethylenimine Analgesic*†

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The fate and metabolism of 4-carbethoxy-1-methyl-4-phenylhexamethylenimine hydrochloride (Wy-401) a synthetic analgesic, was studied in animals with the aid of the C^{14}-labeled drug. Six degradation products were found in the urine; the result of hydrolysis, demethylation, and hydroxylation. Following injection, liver and kidney were found to contain the greatest concentrations of the labeled drug. Highest blood levels were reached within one hour of intra-abdominal injection and within 48 hours the urine was void of all traces of the drug or its metabolites.

IN A RECENT STUDY on the metabolism and excretion rates of meperidine (1) the demethylated and de-esterified derivatives recovered in the urine accounted for one-third of the administered dose. N-demethylation was previously established (2) on the basis of the labeled respiratory CO_2 obtained from animals injected with meperidine tagged in the N-methyl group with carbon 14. A number of phenyl-substituted seven-membered heterocyclic compounds (3, 4) related to meperidine were investigated pharmacologically (5–7) and found to possess analgesic activity.

The present investigation was undertaken to ascertain excretion patterns and products of biotransformation following intra-abdominal administration of one of these drugs, 4-carbethoxy-1-methyl-4-phenylhexamethylenimine hydrochloride (Wy 401). Unlabeled Wy 401 and the drug labeled in the 4- position with carbon 14 were used in this study. Methods were developed for assay of de-esterified metabolites and for a hydroxylated derivative of Wy 401 found in urine.

MATERIALS AND METHODS

Tissue level and excretion pattern studies of Wy 401 and its metabolites were conducted on 14 dogs, 10 rats, and 4 rabbits. The dogs and rabbits were injected intra-abdominally with 35 mg./Kg. and the rats with 50 mg./Kg. of the drug. The urine of dogs and rabbits was collected by catheterization, that of rats in metabolism cages.

Assay of Wy 401 and Its Metabolites.—The determinations of Wy 401 and hydroxy Wy 401 and their de-esterification products were carried out successively on a single 1-ml. urine sample. For every set of analyses, urine collected immediately

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† Ethoheptazine, Wyeth Laboratories.

prior to injection of the drug was treated similarly and served as a blank. In addition, a standard containing 40 mcg./ml. of Wy 401, 40 mcg./ml. of hydroxy Wy 401 and 80 mcg./ml. of de-esterified Wy 401 in preinjection urine was run for comparison. All the determinations involved the methyl orange reaction of Brodie and Udenfriend (8) and were carried out in 15-ml. glass-stoppered centrifuge tubes. As little as 2 mcg. of Wy 401 and hydroxy Wy 401 and 5 mcg. of de-esterified Wy 401 were detected. The methods for extracting Wy 401 and hydroxy Wy 401 before methyl orange complexing involved the same procedures as those used for large scale isolation of the compounds. Specificity of these tests was seen from the infrared, countercurrent and chromatographic analyses of the extracts.

Wy 401.—One milliliter of urine, which had been made alkaline with 0.25 ml. of 1 *N* NaOH, was shaken five minutes with 5 ml. of toluene and centrifuged. The aqueous layer (*A*) was saved for analysis of additional metabolic products, and a 4.5-ml. aliquot of the toluene layer was washed with 2 ml. of 0.2 *M* borate buffer (pH 10). After centrifugation, 4 ml. was removed and shaken with 0.5 ml. of methyl orange reagent (9) and 0.3 ml. isoamyl alcohol. Following centrifugation, 3 ml. of the extract was added to 0.5 ml. of acidified ethanol (2% H_2SO_4 by volume) in a 13 x 75 mm. colorimeter tube. The absorbance was read at 525 mμ in a Fisher electrophotometer. In applying this method to the assay of plasma the wash with borate buffer was omitted. When carried through this procedure, 40 mcg. of Wy 401 in 1 ml. urine gave an absorbance of about 0.77.

Hydroxy Wy 401.—After removing residual toluene, *A* was shaken for five minutes with 5 ml. chloroform to extract hydroxy Wy 401. The aqueous layer (*B*) was set aside and 4.5 ml. chloroform removed and shaken for five minutes with 2.0 ml. 0.2 *M* phosphate buffer (pH 6.0). Four milliliters of the washed chloroform layer was then shaken for five minutes with methyl orange reagent, and 3 ml. of the lower layer was added to the acidified ethanol in a colorimeter tube, and the absorbance read as in the Wy 401 assay.

De-esterified Wy 401 and De-esterified Hydroxy Wy 401.—One-half milliliter *B* was acidified at 0° with 0.5 ml. 50% H_2SO_4. After addition of 5.0 ml. 0.4% $KMnO_4$, the tube was placed in a water bath (Dewar flask) at 53–55° for exactly five minutes, returned to the ice bath and 1 ml. saturated $NaHSO_3$ solution added immediately. After treatment with 1.5 ml. 50% NaOH, the tube was shaken with 5.0 ml. toluene for five minutes and the latter layer carried through as in the Wy 401 assay for plasma. Reproducibility is dependent upon strict adherence to the time and temperature of incubation during the oxidation process. The identity of the material in the toluene layer has not been fully ascertained but the infrared spectrum indicates that the phenyl and carboxyl groups have been removed from the hexamethylenimine ring.

Labeled Wy 401.—To synthesize labeled Wy 401, carboxyl-labeled benzoic acid was first prepared by C^{14}-carbonation[1] of phenylmagnesium bromide (10). The acid was reduced to the alcohol with $LiAlH_4$, the alcohol converted to the bromide with constant boiling HBr, and the bromide treated with KCN to form the methylene-labeled nitrile. This was used in the synthesis of 4-C^{14}-labeled Wy 401 (3, 4), specific activity 0.33 microcuries per mg.

Carbon 14 assay of urine and tissue samples consisted of wet (11) and dry combustion respectively, followed by conversion of the labeled CO_2 to $BaCO_3$ (Li_2CO_3 for samples of low activity) which was then counted at infinite thickness using a thin window Geiger-Mueller counter.

Paper Chromatography.—Urine obtained by catheterization was either spotted (1,000 counts per minute per spot) directly or lyophilized immediately after collection to avoid possible deterioration of samples. Wy 401 and its metabolites were readily extracted from the dried urine samples with 80% ethanol. Since inorganic salts and many of the substances normally present in urine are not extracted by this solvent, sharper separations of metabolites on chromatograms were obtained. The most effective solvent systems for ascending chromatography were butanol saturated with water (12) and butanol saturated with 1 *M* ammonium acetate (pH 7.0). The latter was also used in descending chromatography. Other systems tried were—butanol : glacial acetic acid : water (4:1:5) (13), and isoamyl alcohol saturated with 2% NH_4OH. Paper electrophoretic separations were carried out using 0.05 *M* citrate buffer at pH 5.0.

Indicator sprays included $Bi(NO_3)_3$-KI reagent (14), 0.04% bromcresol green in 95% ethanol, diazotized *p*-nitroaniline (15) and an adaptation of a Feigl spot test for secondary amines (16) which was carried out by first spraying with freshly prepared 2% sodium nitroprusside in 10% acetaldehyde and followed, when the paper was almost dry, with 2% Na_2CO_3.

Radioautographs were made on Kodak No-Screen X-ray film and the chromatographic strips counted by means of an improvised slotted adapter on a Geiger-Mueller counter.

Twelve-inch sheets of Whatman #1 were streaked separately with 1 ml. of urine collected before and after injection with Wy 401. Following development in appropriate systems, narrow lateral strips were removed for spraying with indicators, or scanning if the labeled drug had been injected. Each metabolite band as well as the areas between them were eluted separately, first with 50% ethanol and then with 0.1 *N* NaOH. All eluates were freeze-dried before glucuronide (17), ester and ether sulfate (18), Wy 401, hydroxy Wy 401 and de-esterified Wy 401 determinations were carried out. The corresponding areas of the chromatograms of preinjection urine were eluted and assayed similarly.

RESULTS

Identification of Wy 401 and Its Metabolites in Dog Urine.—When urine from dogs injected with labeled Wy 401 was examined by paper chromatography, the presence of six radioactive substances was shown. Two dimensional chromatography in a variety of systems revealed no additional degradation products. Table I gives the R_f values of the various metabolites in two solvent systems, the pattern on a paper electrophoretogram, and the colors with various indicators. Interference by normal urine constituents was minimal, since two

[1] $BaC^{14}O_3$ purchased from Oak Ridge National Laboratories.

TABLE I.—WY 401 METABOLITES

Metabolite	R_f Values[a]	R_f Values[b]	Electrophoretic migration[c] cm.	Diazotized *p*-nitroaniline	Bismuth iodide	Nitro prusside
1	0.73	0.89	16.2	pale yellow	orange	...
2	0.71	0.73	11.2	pale yellow	orange	...
3	0.41	0.38	7.7	bright yellow	pale yellow	bright blue
4	0.39	0.35	7.6	purple	...	...
5	0.35	0.22	7.5	tan[d]	orange	...
6	0.18	0.11	2.2	brick red	orange	...

[a] Butanol saturated with water.
[b] Butanol saturated with 1 *M* NH_4AcO (pH 7.0).
[c] Sixteen hours, 0.05 *M* (pH 5.0) citrate buffer.
[d] Concealed by substances normally present in urine.

of the indicator sprays [$Bi(NO_3)_3$-KI and nitroprusside] gave color with preinjection urine only at the origin and the other, diazotized *p*-nitroaniline, only to an R_f of 0.35 readily distinguishable from Wy 401 metabolites.

R_f values of substance 1 (Table I) corresponded with those of Wy 401 in the four chromatographic solvent systems used. To isolate unchanged Wy 401, 500 ml. of urine was adjusted to pH 11.0 with 50% NaOH and extracted twice with equal volumes of toluene. The combined extracts were washed twice with equal volumes of borate buffer (pH 10) and then evaporated to dryness. Infrared spectra, R_f values and a 10-tube countercurrent distribution (19) of the dried extract in a phosphate buffer-toluene system (pH 6) were identical with those of authentic Wy 401.

Metabolite 2, tentatively identified as hydroxy Wy 401, was at first thought to be norWy 401, since its R_f values had the same relationship to the parent compound as did normeperidine[1] to meperidine. In order to isolate hydroxy Wy 401, one liter of urine containing this metabolite was adjusted to pH 11.0, washed twice with equal volumes of toluene and once with ether. The pH was then adjusted to 5.5 and the urine again washed with ether. After a final ether wash at pH 7.0, the aqueous phase was twice extracted with equal volumes of chloroform. The combined extracts were dried over anhydrous Na_2SO_4 and the solvent removed under vacuum. The residue was taken up in 10 ml. of acetone, treated with four drops of concentrated HCl to form the hydrochloride, and evaporated to dryness under vacuum. The hydrochloride was recrystallized from acetone-ether. The white crystals (yield, 200 mg.) after drying over phosphorus pentoxide melted at 114°–116° (Fisher Block).

Anal.—Calcd. for $C_{16}H_{25}O_3NCl$: C, 61.23; H, 7.71; N, 4.46; Cl, 11.30. Found: C, 61.06; H, 7.77; N, 4.40; Cl, 11.18.

Failure to obtain authentic samples of either norWy 401 or hydroxy Wy 401 hindered identification of these compounds. However, normeperidine gave a positive nitroprusside test for secondary amines (19) whereas metabolite 2 did not. Furthermore, on the basis of the infrared spectrum which indicated the presence of a hydroxyl group on the hexamethylenimine ring and the chemical composition of metabolite purified by countercurrent extraction, the possibility that this compound was norWy 401 was definitely excluded. Tests on metabolite 2 for ester and ether sulfate, glucuronide, existence of carbonyl, and *p*-hydroxy (9) groups or unsaturation were negative. The infrared spectrum indicated a monosubstituted phenyl ring and an intact ester group in addition to the hydroxyl group. The free base, a white hygroscopic solid melts at 112°–113°. It is insoluble in benzene, ethyl acetate and ether; but soluble in water, butanol and lower alcohols, acetone, and to a certain extent chloroform. The hydrochloride is somewhat less hygroscopic than the free base and has similar solubility characteristics.

Metabolites 3 and 4 have thus far eluded isolation. These substances which appear as a single spot on a radioautograph stain bright yellow and purple, respectively, with *p*-nitroaniline, have the same solubility characteristics as many of the substances normally present in urine, and are extremely labile in both acid and alkali. Eluates of these substances from paper chromatograms had negligible absorbances when complexed with methyl orange in the procedure outlined for complete urine analysis. On the basis of an intense blue color with the nitroprusside spray indicating the presence of a secondary amine, metabolite 3 is presumed to be norWy 401; although the possibility that ring cleavage at the nitrogen atom has occurred, rather than demethylation, is not ruled out.

The identity of metabolite 5 with authentic de-esterified Wy 401 was established by paper chromatography and electrophoresis and by comparison of the infrared spectrum of the esterified metabolite with that of Wy 401. Five hundred milliliters of urine were shaken with toluene and chloroform to remove the parent compound and its hydroxylated derivative before esterification. The urine was lyophilized and then refluxed with absolute ethanol and concentrated H_2SO_4 for four and one-half hours (2). The resulting esterified product was identified as Wy 401. Further transformation of metabolite 5 did not occur; when injected intra-abdominally, it was excreted unchanged by dogs.

Metabolite 6, detected as an orange spot on chromatograms sprayed with bismuth iodide reagent, is hydrolyzed hydroxy Wy 401.

In contrast with results obtained by Plotnikoff, *et al.* (20), using meperidine, none of the metabolites of Wy 401 appeared as conjugates. Incubation of 10-ml. urine samples at pH 5 with 5,000 units of β-glucuronidase (Worthington) did not increase the amount of assayable Wy 401, hydroxy Wy 401, or their de-esterified products. Chromatograms of treated and untreated urine were identical. Moreover, urine collected after injection of the labeled drug did not lose appreciable activity after lead

[1] Generously supplied by Dr. M. L. Tainter, Sterling-Winthrop Institute, Rensselaer, N. Y.

precipitation at pH 4 and pH 8 (21). Results of glucuronide and ether and ester sulfate determinations on paper chromatogram eluates of pre- and postinjection urine samples were identical.

Small quantities of metabolites other than those mentioned were found in ether extracts of acidified urine. This suggests ring cleavage and loss of nitrogen to give acidic substances, such as substituted adipic acids. Ring cleavage is also implied by the relatively low (less than one-third) recoveries of metabolites from urine. Complete breakdown did not occur, however, since the respiratory carbon dioxide of rats injected with labeled Wy 401 was free of carbon 14 (Fig. 1).

Daily doses of Wy 401 for a week had no effect on the relative amounts or types of metabolites found in dog urine.

Effects of Stereoisomerism.—Table II illustrates the contrast in excretion patterns between the *d*- and *l*-forms of Wy 401. Excretion of unchanged Wy 401, hydroxy Wy 401, and their de-esterified products was approximately three times greater after injection of the *d*-form. Furthermore, the average amounts of *d*- and *l*-metabolites closely corresponded to the pattern obtained after injection of racemic Wy 401.

Metabolism of Wy 401 in Other Species.—Following injection of Wy 401 into either rats or dogs the same metabolites appeared in the urine, although at a faster rate in the former species.

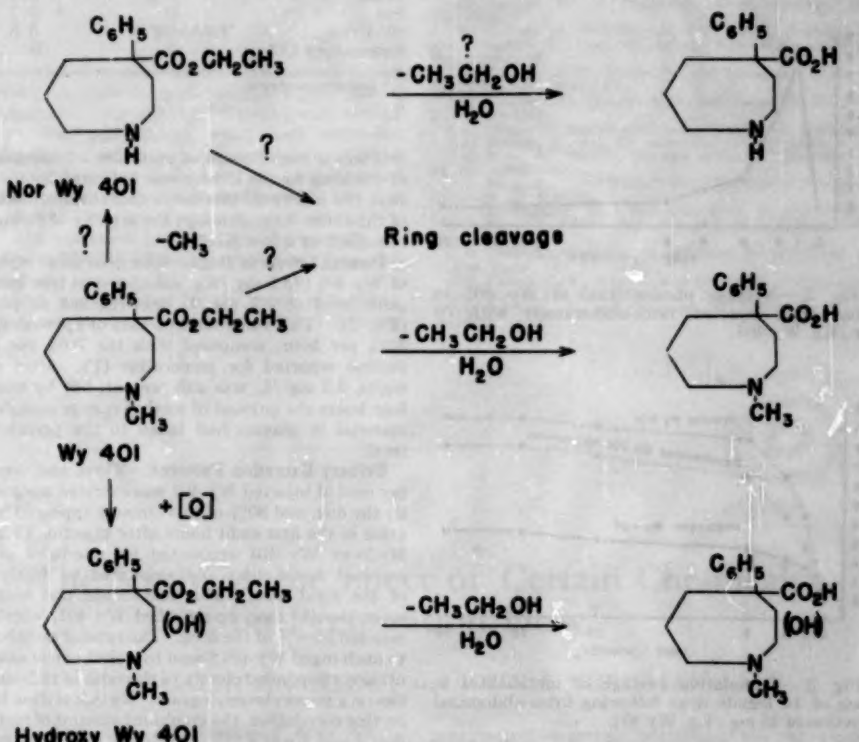

Fig. 1.—Biological degradation of Wy 401.

TABLE II.—EFFECT OF STEREOISOMERISM ON METABOLISM OF WY 401[a]

Metabolite Form Injected	Wy 401 *d*	Wy 401 *l*	Hydroxy Wy 401[b] *d*	Hydroxy Wy 401[b] *l*	Hydrolyzed Wy 401 & Hydroxy Wy 401 *d*	Hydrolyzed Wy 401 & Hydroxy Wy 401 *l*	Total *d*	Total *l*
Amount excreted (per cent of dose)	2.55	0.81	17.49	4.70	12.13	4.92	32.17	10.43
Ratio *d*/*l*	3.14		3.72		2.50		3.08	
(*d* + *l*)/2	1.68		11.10		8.52		21.30	
Excretion following *dl*-Wy 401 injection[c]	3.45		9.34		7.91		20.70	

[a] Results are average of six dogs in each group receiving 35 mg./Kg. of *d*- or *l*-Wy 401 intra-abdominally.
[b] Position of hydroxyl group unknown.
[c] Average of 12 dogs.

Only a trace (0.45%) of Wy 401 was excreted unchanged after intra-abdominal administration to rabbits and a little less than 5% was hydrolyzed. There was also present in rabbit urine a secondary amine, similar to metabolite 3, and a metabolite not present in dog urine.

Distribution of Wy 401 in Rat Tissue.—Two rats were sacrificed thirty minutes after injection of labeled Wy 401, and two after ninety minutes, and the organs assayed for radioactivity (Table III). At thirty minutes, liver had the highest activity, and kidney was next. At ninety minutes, the activity of most organs dropped from one-third to one-half their thirty-minute value, except for intestine which increased four or fivefold; indicating that this was a major route of excretion. Nonretention of the drug by the kidney was indicated by the fact that the ninety-minute urine contained almost 6% of the entire dose, although the activity of this organ remained at a low level.

TABLE III.—DISTRIBUTION OF C^{14} FOLLOWING INTRA-ABDOMINAL INJECTION OF C^{14} WY 401 IN THE RAT

Organ	C^{14} Content of Organ Time of Sacrifice 30 Min. % of Dose	90 Min. % of Dose
Liver	15.6	6.5
Kidneys	3.6	1.9
Lungs	2.0	0.7
Spleen	1.7	0.7
Stomach[a]	1.1	0.4
Testes	1.7	0.8
Intestines[a]	7.0	33.6
Heart	0.3	0.1
Cecum	0.4	0.3
Blood	4.9	0.9
Brain	0.3	0.4
Fat	0	5.2
90′ Urine	...	5.8
Respiratory CO_2	0	0

[a] Includes contents.

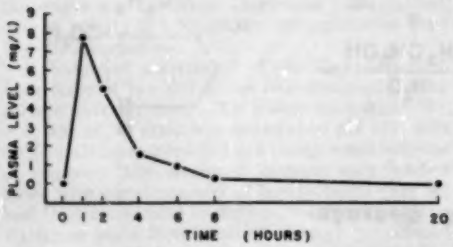

Fig. 2.—Average plasma level of Wy 401 in three dogs injected intra-abdominally with 70 mg./Kg. Wy 401.

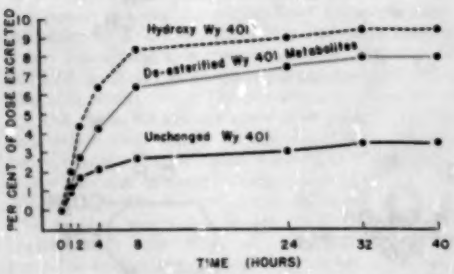

Fig. 3.—Cumulative average of metabolites in urine of 14 female dogs following intra-abdominal injection of 35 mg./Kg. Wy 401.

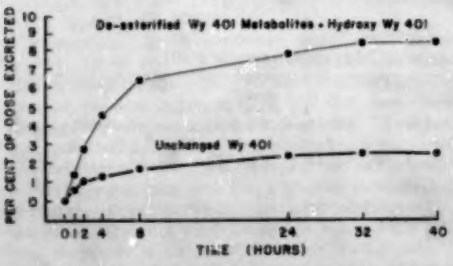

Fig. 4.—Cumulative average of metabolites in urine of 10 male rats following intra-abdominal injection of 50 mg./Kg. Wy 401.

Plasma Levels in Dogs.—One hour after injection of Wy 401 (73.5 mg./Kg. calculated as free base), a peak level of 7.7 mg./L. was reached in plasma (Fig. 2). This value fell at a rate of approximately 35% per hour, compared with the 70% per hour decline reported for meperidine (1). After eight hours, 0.3 mg./L. was still present, but by twenty-four hours the amount of methyl orange complexing material in plasma had fallen to the preinjection level.

Urinary Excretion Patterns.—Three and one-half per cent of injected Wy 401 was excreted unchanged by the dog, and 80% of this amount appeared in the urine in the first eight hours after injection (Fig. 3). Hydroxy Wy 401 accounted for one-third of the excreted metabolites and amounted to nearly 9% of the total dose. This metabolite was excreted more rapidly than de-esterified Wy 401, which accounted for 6% of the dose. The ratio of metabolites to unchanged Wy 401 found by colorimetric assay in urine corresponded closely to the ratio of radioactivities on a paper chromatogram. By calculation based on this correlation, the combined amount of metabolites 3 and 4 was 8.2% of the dose, and de-esterified hydroxy Wy 401 was 1.6% of the dose.

One-third of a dose of hydroxy Wy 401 was excreted unchanged and slightly more than 8% as the hydrolyzed product. This indicated a resistance to further degradation similar to that displayed by de-esterified Wy 401. Of a 35 mg./Kg. dose of the latter administered to three dogs, 40% of the dose was recovered unchanged. Radioautographs indicated negligible biotransformation of this substance.

Ten rats injected with Wy 401 (Fig. 4) excreted approximately 2% of the dose unchanged and 8.4% as hydrolyzed Wy 401 and hydroxy Wy 401.

After forty-eight hours the urine of all animals contained no detectable amounts of Wy 401 or its metabolites.

Meperidine.—Using the methods herein reported, a short study of nonlabeled meperidine was made.

The urine of dogs injected with 35 mg./Kg. meperidine was collected until meperidine and its metabolites could no longer be detected (forty-eight hours). As little as 2 mcg./ml. of meperidine or normeperidine could be detected by the Wy 401 method, but the test could not distinguish between the two compounds. These accounted for 5.3% of the administered dose.

Standards of meperidinic and normeperidinic acids in urine (80 mcg./ml.) had an absorbance of 0.54 when treated by the permanganate method used for assay of de-esterified Wy 401. Five micrograms of either acid could be detected by this method, but again the test could not distinguish between them. These acids and possibly their bound forms (1) accounted for 45% of the injected meperidine.

SUMMARY

The metabolism and excretion pattern of a new analgesic, 4-carbethoxy-1-methyl-4-phenylhexamethylenimine hydrochloride (Wy 401), were studied in dogs, rats, and rabbits, using both unlabeled and the C^{14}-tagged drug. The metabolites differed from that anticipated on the basis of results reported with meperidine in that a hydroxy metabolite was isolated from the urine of animals injected with Wy 401. The excretion pattern and blood levels obtained with Wy 401 differ somewhat from those with meperidine.

Methods are described for the assay of hydroxy Wy 401 and for de-esterified Wy 401. The latter test is applicable to the determination of 1-methyl-4-phenyl-isonipecotic acid (hydrolyzed meperidine).

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The Study of the Effect of Certain Chemicals on Perspiration Flow*

By GALEN F. COLLINS† and JOHN E. CHRISTIAN

A series of new antiperspirant salts was evaluated against currently used products on a percentage and on a molar basis with respect to the cation. In addition, several commercial antiperspirant creams and several newly formulated creams were evaluated. Five of the formulated creams were found to be superior to the most effective commercial antiperspirant products now in use.

TWO TYPES OF SWEAT GLANDS are found in humans, namely the eccrine and apocrine sweat glands. The eccrine sweat glands are distributed over the whole body except on the lip margin and on certain portions of the sex organs. The apocrine glands occur mainly in the axillary regions, but to some extent in the pectoral, abdominal, and genital regions. It is difficult, if not impossible, using the present methods to evaluate antiperspirant preparations on the apocrine glands of humans because of the position of the axillary regions, and because the apocrine sweat glands are mixed with the eccrine sweat glands.

After a method was perfected for the evaluation of antiperspirant preparations on the apocrine sweat glands of the horse (1), several commercial antiperspirant products, a series of antiperspirant salts including several previously un-

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synthesized salts, and several formulated creams were evaluated.

Histological studies of horse skin before and after treatment with antiperspirant preparations were also conducted in an attempt to gain further knowledge concerning their effect on the apocrine sweat glands.

EXPERIMENTAL

The Synthesis of Several Antiperspirant Salts.—Zinc methionate, cerous methionate, cerous sulfamate, and lanthanum sulfamate were synthesized. They were prepared by reacting the appropriate sulfate with barium methionate or barium sulfamate forming barium sulfate which was filtered off, leaving the solution of the desired salt. The salts were isolated and identified (see Table I).

Evaluation of Antiperspirant Preparations.—The following procedure was used for inducing and measuring the perspiration flow on the horse for the subsequent evaluation of antiperspirants. The thoracic regions are readily accessible and contain numerous sweat glands; therefore, these areas were used for the evaluations. Two days prior to the testing procedures, an area 8 by 8 inches was shaved on the thoracic region. The animal was placed in a warm room (temperature 33 to 37°), and the shaven area was wiped with a moist cloth to remove the dirt and hair, and then allowed to dry. Nine areas (2 by 2 inches), arranged in three vertical rows, were used for the evaluations. Three specially prepared adhesive tape holders containing three equally spaced disks of absorbent blotting paper were taped in a vertical position over the nine areas. Each holder consisted of three layers of Curity Wet Pruf adhesive tape 2 by 6 inches with three holes, 28 mm. in diameter, evenly spaced on

TABLE I.—DESCRIPTION AND ANALYSIS OF ANTIPERSPIRANT SALTS

Salt[a]	Approximate pH	Analyzed for	Analysis Calcd., %	Found, %
$ZnCH_2(SO_3)_2 \cdot 3H_2O$[b]	(25% Solution) 4.3	Zn	22.27	22.24
		H_2O	18.39	18.36
$Ce_2[CH_2(SO_3)_2]_3 \cdot 6H_2O$[c]	(25% Solution) 4.5	Ce	30.72	30.74
		H_2O	11.86	11.83
$La(H_2NSO_3)_3 \cdot 3H_2O$[c,d,e]	(20% Solution) 1.7	La	28.87	28.76
		S	19.98	19.92
$Ce(H_2NSO_3)_3 \cdot 3H_2O$[e, f]	(20% Solution) 2.0	Ce	29.04	28.94
		S	19.94	20.15

[a] All the salts are colorless and are readily soluble.
[b] This salt was isolated by adding a large excess of 25% ethyl alcohol–75% acetone mixture to a concentrated aqueous solution.
[c] These salts were isolated by adding a large excess of ethyl alcohol to a concentrated aqueous solution.
[d] This salt has been previously prepared by Kleinberg, *et al.* (2), using a different procedure.
[e] These compounds are unstable in hot aqueous solutions forming the sulfate ion plus other unidentified substances.
[f] This salt was isolated by concentrating an aqueous solution under reduced pressure at room temperature.

TABLE II.—THE RELATIVE ANTIPERSPIRANT EFFECTIVENESS OF SOME SALT SOLUTIONS WHEN COMPARED TO A 24% (W/W) SOLUTION OF $Al_2(SO_4)_3 \cdot 18H_2O$

Solution[a]	Relative[b] Effectiveness	Approx. pH of Solution	Solution	Relative Effectiveness	Approx. pH of Solution
25% $Al(H_2NSO_3)_3$	1.98 ± 0.35[c]	2.3	20% $Ce(H_2NSO_3)_3 \cdot 3H_2O$	1.36 ± 0.24	2.0
25% $Al_2(CH_2(SO_3)_2)_3$	1.95 ± 0.35	3.2	48% Sodium Zirconium Lactate[f]	0.67 ± 0.03	8.4
16% $Al_2(CH_2(SO_3)_2)_3$	1.31 ± 0.15	3.0	.....	...	..
25% $AlCH(SO_3)_3$	1.07 ± 0.08	3.3	.....	...	..
15% $AlCl_3 \cdot 6H_2O$	1.06 ± 0.15	2.2	.....	...	..
15% $Al_2(SO_4)_3 \cdot 18H_2O$	0.48 ± 0.07	2.8	.....	...	..
20% Aluminum Chlorhydroxide Complex	0.31 ± 0.02	4.1	12.5% $La_2(CH_2(SO_3)_2)_3 6H_2O$) 12.5% $Ce_2(CH_2(SO_3)_2)_3 6H_2O$)	2.60 ± 0.46	4.0
25% $La(NO_3)_3 \cdot 6H_2O$[d]	3.01 ± 0.85	3.3	.....	...	..
25% $La_2(CH_2(SO_3)_2)_3 \cdot 6H_2O$	2.81 ± 0.40	4.4	.....	...	..
25% $Ce_2(CH_2(SO_3)_2)_3 \cdot 6H_2O$	2.58 ± 0.55	4.5	12.5% $Al_2(CH_2(SO_3)_2)_3$ 12.5% $Ce_2(CH_2(SO_3)_2)_3 \cdot 6H_2O$	1.98 ± 0.37	3.2
25% $ZnCH_2(SO_3)_2 3H_2O$[e]	2.20 ± 0.64	4.3	Untreated Area	0.37 ± 0.08	
20% $La(H_2NSO_3)_3 \cdot 3H_2O$	1.89 ± 0.40	1.7			
23% $LaCl_3 \cdot 7H_2O$	1.43 ± 0.19	5.2	24% $Al_2(SO_4)_3 \cdot 18H_2O$		2.6

[a] All the solutions were (w/w) and contained 0.2% Tween 20.
[b] The relative effectiveness of the solution was obtained by dividing an average of 8 treated ratios by the average of 8 normal ratios. A value greater than 1 indicates that the various solutions were more effective than aluminum sulfate solution. For example, the 25% $Al(H_2NSO_3)_3$ solution was 1.98 times more effective than the 24% $Al_2(SO_4)_3 \cdot 18H_2O$ solution.
[c] This value is the standard error of the relative effectiveness at the 0.05 level.
[d] These solutions were very irritating to the skin as shown by considerable sloughing of the epidermis.
[e] These solutions were irritating as shown by some sloughing of the epidermis.
[f] This solution was obtained from the National Lead Company.

TABLE III.—THE RELATIVE ANTIPERSPIRANT EFFECTIVENESS OF SOME SALTS ON A MOLAR BASIS WITH RESPECT TO THE CATION WHEN COMPARED TO A 0.25 *M* ALUMINUM SULFATE SOLUTION

Solution[a]	Relative Effectiveness[b]	Approximate pH of Solution
0.5 *M* (24.06%)		
$La(H_2NSO_3)_3 \cdot 3H_2O$	4.86 ± 1.96[c]	1.7
0.25 *M* (22.7%)		
$La_2(CH_2(SO_3)_2)_3 \cdot 6H_2O$	3.36 ± 0.74	4.7
0.25 *M* (22.75%)		
$Ce_2(CH_2(SO_3)_2)_3 \cdot 6H_2O$	2.84 ± 0.52	4.8
0.5 *M* (24.12%)		
$Ce(H_2NSO_3)_3 \cdot 3H_2O$	2.46 ± 0.10	1.9
0.5 *M* (18.57%)		
$LaCl_3 \cdot 7H_2O$	2.19 ± 0.32	4.8
0.5 *M* (12.07%)		
$AlCl_3 \cdot 6H_2O$	2.13 ± 0.36	2.4
0.5 *M* (14.67%)		
$ZnCH_2(SO_3)_2 \cdot 3H_2O$[d]	2.10 ± 0.46	4.5
0.5 *M* (15.71%)		
$Al(H_2NSO_3)_3$	2.07 ± 0.30	2.7
0.5 *M* (15.35%)		
$ZnSO_4 \cdot 7H_2O$[d]	1.79 ± 0.31	5.8
0.25 *M* (14.4%)		
$Al_2(CH_2(SO_3)_2)_3$	1.76 ± 0.15	3.3
0.5 *M* (14.9%)		
$AlCH(SO_3)_3$	1.09 ± 0.10	3.6
0.25 *M* (16.6%)		
$Al_2(SO_4)_3 \cdot 18H_2O$	...	2.9

[a] The percentages of these solutions are given on a (w/v) basis. All the solutions were 0.5 *M* with respect to cation and contained 0.2% Tween 20 and 8% urea.

[b] The relative effectiveness of the solution was obtained by dividing an average of 8 treated ratios by the average of 8 normal ratios. A value greater than 1 indicates that the various solutions were more effective than the aluminum sulfate solution; for example, the lanthanum sulfamate solution is 4.86 times more effective than the aluminum sulfate solution.

[c] This value is the standard error of the relative effectiveness at the 0.05 level.

[d] These solutions were slightly irritating to the skin as shown by a slight amount of sloughing of the epidermis.

the bottom two layers of the adhesive tape. Round pieces of rubber dam, 32 mm. in diameter, were held over each of the holes by the top layer of adhesive tape. When the adhesive tape holder was taped on the animal, three airtight receptacles were provided for three disks of 100-pound glazed blotting paper 28 mm. in diameter. The absorbent side of the glazed blotting paper was always faced toward the skin of the animal. Elastic bands two inches wide were strapped around the trunk of the animal over the adhesive tape holders to hold them tightly against the skin and to prevent them from slipping out of position. To induce sweating, a large piece of rubber dam was placed on the back of the horse, followed by two heavy blankets which were strapped around the trunk of the animal. The entire trunk of the animal was covered, except for a narrow strip extending through the center of the abdominal region. The blankets were kept on the horse for about fifteen minutes to obtain a sufficient amount of sweat for an evaluation (35 mg. to 55 mg./area). The blankets and adhesive tape holders were removed and the disks of blotting paper immediately placed in weighing bottles and weighed. The increase in weight over the previously tared disks and weighing bottles gave the quantity of sweat secreted from each respective area. When another test was conducted immediately, the areas were thoroughly dried by wiping with a dry towel.

The normal sweating ratios between the untreated areas were determined by conducting four tests a day for two days. The antiperspirant preparations (90 mg. of creams and 0.07 ml. of solutions) were applied to the respective areas immediately after the normal sweating ratios were completed and twice daily for the next six days. The preparations were evaluated by comparing their antiperspirant effectiveness to a standard cream or solution. The standard cream or solution was applied to the center area

TABLE IV.—THE RELATIVE ANTIPERSPIRANT EFFECTIVENESS OF COMMERCIAL ANTIPERSPIRANT PRODUCTS AND SOME NEWLY FORMULATED ANTIPERSPIRANT CREAMS

Preparations Tested, Commercial Products[a]	Relative[b] Effectiveness	Approx. pH	Preparations Tested, Formulated Creams	Relative Effectiveness	Approx. pH
A	Standard[c]	3.3	24% (w/w) $Al_2(SO_4)_3 \cdot 18H_2O$	Standard[f]	2.6
B	3.10 ± 0.66[d]	2.7	Product C[g]	1.59 ± 0.27	2.7
C	3.42 ± 0.82	2.7	25% Lanthanum Sulfamate	2.48 ± 0.06	5.4
D	0.74 ± 0.03	3.8	25% Lanthanum Methionate[h]	2.42 ± 0.12	5.7
E	1.57 ± 0.29	3.0	25% Cerium Methionate	2.31 ± 0.09	5.3
F	1.06 ± 0.11	3.0	25% Cerium Sulfamate	1.80 ± 0.07	5.3
G	0.80 ± 0.11	*	25% Aluminum Methionate	1.85 ± 0.24	2.7
H	1.13 ± 0.03	3.2	25% Zirconium Methionate[h] Tetrahydrated	1.24 ± 0.04	5.8
I	0.79 ± 0.05	3.9	.....	...	..
J	0.71 ± 0.09	3.6	.....	...	..

[a] The active ingredients of the commercial products include: aluminum chloride, aluminum sulfate, aluminum sulfamate, aluminum chlorhydroxide complex, aluminum phenolsulfonate, and basic aluminum formate.

[b] The relative effectiveness was obtained by dividing the average of 8 treated ratios by the average of 8 normal ratios.

[c] The commercial products were evaluated by comparing their antiperspirant effectiveness to that of Product *A*. A value greater than 1 indicates that the products are more effective than Product *A*. For example, Product *B* is 3.1 times more effective than Product *A*.

[d] This is the standard error of the relative effectiveness at the 0.05 level.

[e] The pH of this preparation could not be readily taken since it is a water-in-oil emulsion.

[f] The formulated antiperspirant creams were evaluated by comparing their antiperspirant effectiveness to that of a 24% (w/w) solution of $Al_2(SO_4)_3 \cdot 18H_2O$. A value greater than one indicates that the preparations are more effective than the aluminum sulfate solution; for example, the lanthanum sulfamate cream is 2.48 times more effective than the aluminum sulfate solution.

[g] Product *C* was compared to the aluminum sulfate as a basis for comparing the most effective commercial product to the newly formulated creams.

[h] The lanthanum methionate and zirconium methionate were synthesized by Dr. G. E. Cwalina, Pharmaceutical Chemistry Department, Purdue University.

of each vertical row and the preparations being evaluated were applied to the adjacent areas. Four tests were conducted on the sixth and seventh days of treatment to determine the treated sweating ratios. The treated sweating ratios were calculated by dividing the weight of sweat obtained from the areas treated with the standard cream or solution by the weight of sweat obtained from the areas treated with the preparations being evaluated. The relative effectiveness of an antiperspirant preparation was calculated by dividing the average of eight treated sweating ratios by the average of eight normal (untreated) sweating ratios. A method of statistical analysis described by Burn (3) was used to calculate the standard error of the relative effectiveness.

The antiperspirant salts were evaluated on a percentage (w/w) basis and on a molar basis with respect to the cation. The salts evaluated on a percentage basis were compared to a 24% (w/w) solution of $Al_2(SO_4)_3 \cdot 18H_2O$, and the salts evaluated on a molar basis with respect to the cation were compared to a 0.25 *M* aluminum sulfate solution (0.5 *M* aluminum ion). The molar solutions all contained 8% urea to help reduce the acidity of the more acid salts. All the solutions contained 0.2% Tween® 20 to aid in spreading the solutions evenly over the skin. The relative antiperspirant effectiveness of the salts evaluated on a percentage basis are given in Table II, and on a molar basis with respect to the cation are given in Table III.

The commercial products were evaluated by comparing their antiperspirant effectiveness to one of the commercial products, Product *A*, and the formulated creams were evaluated by comparing their effectiveness to a 24% (w/w) solution of $Al_2(SO_4)_3 \cdot 18H_2O$. The ointment base used for the formulated creams contained the following ingredients in variable proportions: Tegacid,[1] spermaceti, sodium lauryl sulfate, glycerin, water, urea, and titanium dioxide. Zinc oxide was added to all the creams except the aluminum methionate cream to reduce the acidity. Zirconium methionate is very acidic (the pH of a 25% (w/w) solution is about 0.7), and it could not be tested in solution form, but when the zirconium methionate was incorporated in a base with 2% zinc oxide, the acidity was reduced considerably. The relative antiperspirant effectiveness of these commercial products and formulated creams are given in Table IV.

Several of the same solutions and creams were evaluated using the same procedures on a different horse, and comparable results were obtained.

Histologic Studies.—Six areas (2 by 2 inches) were selected on the thoracic region for these studies. Starting two days after the areas were shaved, a 24% (w/w) solution of $Al_2(SO_4)_3 \cdot 18H_2O$, a 15% (w/w) solution of $AlCl_3 \cdot 6H_2O$, a 16% solution of $Al_2[CH_3(SO_3)_2]_3$, a 23% solution of $LaCl_3 \cdot 7H_2O$, and a 15% (w/w) solution of $ZnSO_4 \cdot 7H_2O$ were applied to separate areas. One untreated area served as the control. The solutions (0.07 ml.) were applied to each respective area twice daily for seven days.

After seven days of treatment, visual examinations showed that the aluminum chloride and lanthanum chloride solutions caused severe irritation to the skin resulting in considerable sloughing of the epidermis. The zinc sulfate solution caused some irritation as indicated by a slight amount of sloughing of the epidermis.

Biopsy specimens were removed from each area and microscopic slides prepared from each specimen for examination.[2] The sections were stained with eosin and hematoxylin.

Examination of the tissue sections revealed that in a portion of the apocrine sweat glands, all the salts caused atrophy of the secreting cells with pycnosis of some of the nuclei of these cells. In certain cells there was a complete disappearance of the nuclei. There was no evidence of narrowing of the sweat ducts. The aluminum chloride, lanthanum chloride, and zinc sulfate solutions also caused an edematous reaction in the corium. These noted effects with the antiperspirant salt solutions are similar to those found by Sulzberger, *et al.* (5), with an aluminum sulfate cream on the apocrine sweat glands of humans. The degeneration of the secreting cells is undoubtedly responsible, in part, for the reduction in perspiration flow.

Discussion.—On a molar basis with respect to the cation, lanthanum salts were the most effective salts, followed by cerium salts, and then by aluminum salts. This follows the Hofmeister series (4) for trivalent metals, which indicates that this series can be used to predict the antiperspirant effectiveness of the other trivalent metals of this series. Accordingly, some of the trivalent metals can be arranged in the following order starting with the most effective antiperspirant metal: lanthanum, cerium, prasedymium, neodymium, samarium, europium, yttrium, scandium, and aluminum.

Lanthanum, cerium, praseodymium, neodymium, samarium, and europium are members of the Lanthanide Series of the Periodic Table and, since all the members of this series are chemically related, the other members of this series should show antiperspirant activity.

SUMMARY AND CONCLUSIONS

1. Methods were developed for the synthesis of cerous sulfamate, lanthanum sulfamate, cerous methionate, and zinc methionate.

2. A procedure was developed for the evaluation of antiperspirant preparations on the apocrine sweat glands of the horse.

3. A series of antiperspirant salts was evaluated on a percentage basis and on a molar basis with respect to the cation. Since the action of antiperspirants is largely attributed to the cation, the evaluation on a molar basis was considered to be more valid. The relative effectiveness of the salts evaluated on a molar basis is arranged in the following order starting with the most effective salt: lanthanum sulfamate; lanthanum methionate; cerous methionate; cerous sulfamate; lanthanum chloride, aluminum chloride, zinc methionate, and aluminum sulfamate; zinc

[1] An acid stable glyceryl monostearate, Goldschmidt Chemical Corporation, New York City, N. Y.

[2] The slides were prepared by the Pathology Department of St. Elizabeth Hospital, Lafayette, Ind.

sulfate and aluminum methionate; aluminum methane sulfonate and aluminum sulfate.

4. Several commercial antiperspirant creams and several newly formulated antiperspirant creams were evaluated. Five of the formulated creams were found to be superior to the most effective commercial antiperspirant product. The relative effectiveness of the five creams is arranged in the following order starting with the most effective cream: lanthanum sulfamate cream, lanthanum methionate cream, cerous methionate cream, aluminum methionate cream, and cerous sulfamate cream.

5. Histologic examinations were made on the apocrine sweat glands of the horse after several antiperspirant salt solutions were applied twice daily for seven days.

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The Synthesis and Aerosol Formulation of Certain Rare Earth Compounds as Potential Antiperspirants*

By ROGER MANTSAVINOS and JOHN E. CHRISTIAN

Neodymium methionate, praseodymium methionate, samarium methionate, and praseodymium sulfamate have been prepared and characterized. Two new potential antiperspirant mixtures, a mixed rare earth methionate and a mixed rare earth sulfamate, have been synthesized and analyzed. Aerosol formulas of a mixed rare earth methionate and a mixed rare earth sulfamate have been developed and studied for such properties as stability and solubility at various temperatures, vapor pressure, and corrosion.

SWEAT IS THE MOST IMPORTANT SECRETION of one of the largest organs of the human body, the skin. It performs many important physiological functions vital to the well-being of the body.

At times, however, it becomes desirable to temporarily check profuse perspiration on body surface areas with poor ventilation such as the axillary areas. Excessive perspiration in these areas may be corrosive to fabrics, cause skin irritation, and give rise to the social annoyance of offensive odors.

To combat some of these undesirable features, antiperspirants are commonly used. Antiperspirants are usually metallic salts the exact physiological action of which has not been clearly established. Aluminum salts are probably the most extensively used at the present time.

Collins (1) synthesized and introduced certain rare earth methionates and sulfamates as effective antiperspirants. He found solutions and creams of lanthanum and cerium methionate to be more effective than solutions and creams of commonly used commercial antiperspirants when tested on the sweat glands of the horse. Lanthanum sulfamate and methionate were the most effective of the rare earth salts prepared and tested. The relative effectiveness of the trivalent cations of the salts studied by Collins was found to be in accordance with the Hofmeister (2) or lyotropic series. This series is based on the coagulative effects of ions on lyophilic sols, such as egg albumin suspended in water. Kunin and Meyers (3) list the following order for trivalent ions: Al < Sc < Y < Eu < Sm < Nd < Pr < Ce < La. Collins suggested that such a series could be used to predict the effectiveness of the trivalent metals of this series as antiperspirants. Thus, lanthanum methionate would be more effective than cerium methionate which would be more effective than praseodymium methionate, etc. This

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TABLE I.—DESCRIPTION AND ANALYSIS OF RARE EARTH COMPOUNDS

Compound[a]	Color	Analysis: Rare Earth, % Calcd.	Rare Earth, % Found	Water, % Calcd.	Water, % Found
$Nd_2[CH_2(SO_3)_2]_3 \cdot 6H_2O$	Pink	31.40	31.34	11.75	11.63
$Pr_2[CH_2(SO_3)_2]_3 \cdot 6H_2O$	Light green	30.89	30.16	11.84	11.92
$Sm_2[CH_2(SO_3)_2]_3 \cdot 6H_2O$	Light yellow	32.30	32.37	11.60	11.40
$Pr_2[SO_2NH_2]_6 \cdot 3H_2O$[b]	Light green	29.17	29.16	11.18	9.89[c]
$RE_2[CH_2(SO_3)_2]_3 \cdot xH_2O$ (Rare earth methionate mixture)	Pink	...	34.84[d]	...	11.44
$RE_2[SO_2NH_2]_6 \cdot xH_2O$ (Rare earth sulfamate mixture)	Pink	...	34.06[e]	...	...

[a] These compounds are readily soluble in water but are insoluble in the common organic solvents.
[b] Analyzed for sulfur, calcd.: S, 19.91%; Found: S, 19.45%.
[c] Praseodymium sulfamate lost an equivalent of slightly more than 2.5 molecules of water of crystallization before decomposing at 105°.
[d] This value is the total per cent of the rare earth content expressed on an oxide basis. It represents approximately 16.7% of cerium oxide, (CeO_2); 8.4% of lanthanum oxide, (La_2O_3); 6.7% of neodymium oxide, (Nd_2O_3); 2.1% of praseodymium oxide, (Pr_6O_{11}); 0.7% of samarium oxide, (Sm_2O_3); and 0.3% other rare earth oxides, when calculated to the nearest tenth of a per cent.
[e] This value is the total per cent of rare earth content expressed on an oxide basis. It represents approximately 16.4% of cerium oxide, (Ce_2O_3); 8.2% of lanthanum oxide, (La_2O_3); 6.5% of neodymium oxide, (Nd_2O_3); 2.0% of praseodymium oxide, (Pr_6O_{11}); 0.7% of samarium oxide, (Sm_2O_3); and 0.3% other rare earth oxides.

was actually the case with the salts tested by Collins on the sweat glands of the horse.

Christian and Jenkins (4) reported the methionate acid radical to be harmless to fabrics, and Jenkins (5) found it to be nontoxic to the skin.

In view of these facts, the immediate objective of this investigation was to prepare new rare earth methionate and rare earth sulfamate compounds in an endeavor to obtain active antiperspirants devoid of unwanted actions common to many antiperspirants.

Due to the steadily increasing popularity of the aerosol type of product, some of these compounds were formulated into a product of this type.

EXPERIMENTAL

Synthesis of Methionates and Sulfamates.—Three new rare earth methionate compounds—neodymium methionate, praseodymium methionate, and samarium methionate—and one new rare earth sulfamate compound, praseodymium sulfamate, were prepared. In addition, a new rare earth methionate mixture and a new rare earth sulfamate mixture, consisting of the methionates or sulfamates of various rare earths, were prepared from a commercially available mixture of various rare earth sulfates.[1]

These compounds were prepared by reacting a slight excess of barium methionate (6, 7) or barium sulfamate (8) with the corresponding sulfate[2] for forty-eight hours. The reaction mixture was heated to just below the boiling temperature in the syntheses of the methionates, but the reaction was carried out at room temperature in the case of the sulfamates, since the sulfamates were unstable in hot aqueous solutions. The barium sulfate was filtered off and the filtrate concentrated by boiling, if permissible, or under reduced pressure at room temperature. The compound was then separated from the aqueous medium by evaporating to dryness under reduced pressure or by adding either an excess of acetone, or a 1:1 solution of ethyl alcohol (95%) and acetone. The salts were reprecipitated from water, dried, and analyzed. Analyses of the compounds synthesized are shown in Table I.

Aerosol Formulation.—Compatible aerosol formulas of the rare earth methionate mixture and the rare earth sulfamate mixture were studied.

In order to visually observe the various aerosol formulas periodically for signs of incompatibility, the formulas were placed in round bottom, glass, pressure flasks[3] having a volume of 200 ml. These flasks were loaded with the concentrate (formula minus the propellent) and after cooling at 5° for one hour the propellent, which was stored at −22°, was carefully added and the flasks quickly stoppered by clamping down the patent top of the flasks as soon as convection currents of the propellent were seen escaping from the mouth of the flask.

Standard aerosol containers, modified by soldering a threaded bushing on the bottom of the container through which fillings could be made, were also loaded in a similar manner. After loading, the bushing was tightly sealed with a threaded plug.

The propellents used in this study were various Freons®[4] and Genetron®[5] 101. Since the active ingredients were insoluble in the propellents used in this study, a suitable co-solvent was used in which the active ingredient and propellent were mutually soluble. Many organic solvents were disqualified for this purpose either because of their toxicity, poor solvent power, or other undesirable

[1] Supplied from the Lindsay Chemical Co., and Rare Earths, Inc. A representative analysis of the total rare earth content expressed on an oxide basis is as follows: cerium oxide, (CeO_2) 21.6%; lanthanum oxide, (La_2O_3), 10.8%; neodymium oxide, (Nd_2O_3), 8.6%; praseodymium oxide, (Pr_6O_{11}), 2.7%; samarium oxide, (Sm_2O_3), 0.9%; other rare earth oxides, 0.4%. Analysis was supplied by the Lindsay Chemical Co.

[2] Sulfates of 98% or 99% purity were purchased from the Lindsay Chemical Co. for the syntheses of the pure methionate and sulfamate compounds.

[3] Commercially available from the E. H. Sargent Co., Chicago, Ill.

[4] Trademark for propellents sold by the E. I. duPont de Nemours and Co., Inc.

[5] Trademark for propellents sold by the General Chemical Co.

TABLE II.—COMPOSITION OF AEROSOL FORMULAS 1 AND 2

Ingredients	Formula 1, % w/w	Formula 2, % w/w
Rare earth mixture	4.45[a]	4.50[b]
Distilled water	38.22	31.46
Ethyl alcohol (95%)	47.22	53.93
Propellent	10.11	10.11
Rare earth mixture without propellent	4.95[a]	5.0[b]

[a] Mixed rare earth methionate.
[b] Mixed rare earth sulfamate.

formula to be tested and stored for one month at 55° in the case of Formula 1, and at room temperature in the case of Formula 2. No visible evidence of corrosion was found with Formula 1. This is a rather unusual finding when one considers the severe corrosion problem usually encountered in aerosol antiperspirant formulas. Two controls without the mixed rare earth methionate but consisting of the same ratio of alcohol, water, and propellent showed definite signs of corrosion. Unlike Formula 1, Formula 2, containing rare earth sulfamate as the active ingredient, showed signs of mild corrosion at room temperature after a period of one month. Further quantitative tests, however, are

TABLE III.—STABILITY, SOLUBILITY, AND PRESSURE PROPERTIES OF FORMULAS 1 AND 2

Propellent	Formula 1, Solubility[a] 5°	24°	55°	Pressure, lbs./in.[2][b] Absolute	Gage	Formula 2, Solubility 5°	24°	55°
Freon-114	I	I	I	..	..	I	I	S
Freon-12 (50%) and Freon-11 (50%)	I	S[c]	S[c]	49.7	35.0	S[d]	S[d]	S
Freon-12 (50%) and Freon-114 (50%)	I	S	S	54.7	40.0	S	S	S
Genetron 101	S	S	S	45.7	31.0	S	S	S

[a] I—Indicates the propellent (10.11% w/w) was insoluble in the formula, when tested for 24 hrs. S—Indicates the propellent was soluble in the formula in a concentration of 10.11% by weight when tested for 24 hrs.
[b] Pressure measured after formula was kept in a room, air conditioned at 23°, for 24 hours. The pressures measured for Formula 2 were approximately the same in each case.
[c] Also tested for a period of one month at this temperature. At 55° there was a slight discoloration of the solution after three weeks, but no evidence of insolubility of any of the components. There was no evidence of insolubility at 23°.
[d] Also tested at this temperature for one month. No evidence of insolubility after this period of time.

properties. A hydroalcoholic solvent was chosen since this type of solvent is frequently used in aerosol antiperspirant formulas. Formulas with a salt concentration ranging from 5 to 15% (w/w) in various alcohol and water ratios were tried. In all cases, the solubility of the salt decreased and the solubility of the propellent increased with an increase in the alcohol content. Table II gives the formulas found to be most satisfactory.

Various propellents were tested in those formulas for solubility and stability at various temperatures. In addition, the vapor pressures developed by the formulas were measured using a pressure gage screwed into a bushing soldered on the top of a crimped aerosol container. The results of these studies are given in Table III.

The vapor pressures of both formulas, measured by the above method, meet the pressure specifications of the Interstate Commerce Commission (9) for low pressure aerosols.

As a point of interest, the corrosive action of Formulas 1 and 2 was determined qualitatively on high purity aluminum and aluminum 2S (an alloy) using a mixture of Freon-11 (50%) and Freon-12 (50%) as the propellent in each case. Strips of these metals were placed in pressure flasks with the necessary to accurately establish corrosive action.

The pH of an antiperspirant product is an important physical property when considering its practical utilization. The pH values of 5, 10, and 20% (w/w) rare earth methionate solutions were 4.6, 4.5, and 4.4 respectively. The pH of Formula 1, without the propellent, was 4.0. These values compare more than favorably with the pH values of some commonly used antiperspirants. The pH values of 5, 10, and 20% (w/w) solutions of rare earth sulfamate were 1.9, 1.7, and 1.6 respectively; whereas, the pH of Formula 2 without the propellent was 2.4.

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A Study of Dispersion with Ultrasound I*

By BERNARD MISEK† and DONALD M. SKAUEN‡

The suitability of ultrasonic energy as a mechanical dispersing agent in the preparation of progesterone suspensions was investigated. A turbidimetric method was devised to determine the efficiency of ultrasound in reducing the particle size of the suspended material. The addition of a surface-active agent to the suspending liquid was found necessary to obtain a satisfactory dispersion. The concentration of the dispersed material had no effect upon the resulting particle size. The final particle size was directly proportional to the initial particle size, after a standard time of ultrasonic exposure. Maximum dispersion was attained by insonation for a period of fifteen to twenty minutes when 200-mesh progesterone was used. The use of progesterone of a larger initial size increased the time required to produce maximum dispersion. Once maximum dispersion was attained, insonation for longer periods of time produced no measurable effect. Dispersion increased as the ultrasonic intensity was increased.

SEVERAL METHODS are available for the manufacture of pharmaceutical suspensions of optimum particle size. The usual procedures involve the use of mills, such as the ball mill or colloid mill, which reduce the size of material suspended in a liquid medium. Other types of mills, such as the micronizer, reduce the size of solid materials which are then dispersed in a liquid medium.

In recent years a new mechanical dispersing method has been introduced whereby dispersion and particle size reduction of the dispersed phase is obtained by means of ultrasonic energy. The dispersion of dyestuffs and pigments by ultrasonic vibrations has been reported in the literature (1–3). The dispersion of metal hydroxides (4), carbon in aqueous alcohol (5), and a vanadium pentoxide sol (6) have also been noted. Microcrystals of sulfathiazole have been prepared using sound energy (7). Seidl (8) has demonstrated the preparation of colloidal gold suspensions by the action of ultrasound with the resulting suspensions being free from the usual toxic reactions of other gold preparations.

This study was undertaken to investigate the suitability of employing ultrasonic energy for dispersion and particle size reduction in the manufacture of pharmaceutical suspensions and to evaluate the important factors in such a process.

EXPERIMENTAL METHODOLOGY

Progesterone was selected as the material to be dispersed since the preparation of its aqueous suspensions is a problem in the pharmaceutical industry. This is due to the difficulty of reducing the crystal size of progesterone which is available in the form of relatively large crystals (9).

The ultrasonic generator used in this investigation was a General Electric model G-3 which transforms high frequency electrical oscillations into ultrasonic vibrations by means of a quartz crystal. A stainless steel chamber[1] with a bottom consisting of an acoustically transparent stainless steel diaphragm was employed for exposing samples to the ultrasonic irradiation. Approximately 250 cc. of a 0.05% aqueous solution of Tween 80 was used as the coupling fluid in the sound chamber.

Except where otherwise indicated, all samples were exposed to ultrasonic irradiation for fifteen minutes at a frequency of 300 kc./sec. The oscillator plate power was set at 175 ma. producing approximately 57.5 watts of acoustical energy in the sound chamber, as determined calorimetrically. The distance between the base of the exposure chamber and the top of the vibrating crystal was 34 mm. Each sample contained 100 mg. of progesterone, 200 mesh, dispersed in 10 cc. of a 0.1% aqueous Superinone solution.

A turbidimetric method was devised for the evaluation of the effect of ultrasound upon the particle size reduction of the suspended material. The first step in developing a method applicable to this investigation was to determine the relationship between the projected surface area of the suspended particles and the absorbance readings. This was done by taking absorbance measurements of increasing concentrations of suspended progesterone. The results obtained, as shown in Fig. 1, establish the fact that the absorbance of these suspensions varies linearly with the concentration of the suspended matter and therefore with the surface area.

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[1] Ultrasonic Engineering Company, Maywood, Ill.

Thus, by keeping the concentration of the suspended material constant, the absorbance of the suspensions will vary with the projected surface area and hence with the particle size.

In order to correlate the absorbance readings with the weight distribution of the progesterone particles, particle size distribution counts were made on several suspensions having a fixed concentration of suspended progesterone but different absorbances. The absorbance was, therefore, a function of the particle size. The results obtained were then converted to give the relative-weight distribution according to particle-size ranges and are reported, along with the absorbance readings, in Table I.

EXPERIMENTAL

Effect of Ultrasound on Stability of Progesterone Molecule.—It has been shown (10) that ultrasonic energy can cause the decomposition of various compounds. In view of this fact it was decided to study the effect of ultrasonic vibrations on the stability of progesterone.

A 10-cc. sample of suspension containing 10 mg. of progesterone per cc. of a 0.1% Tween 80 solution was exposed to ultrasonic irradiation for forty-five minutes with the acoustical energy in the sound chamber set at approximately 70 watts.

TABLE I.—DISTRIBUTION OF PROGESTERONE IN SUSPENSIONS OF VARYING ABSORBANCE

	Absorbance of Suspensions					
Class Boundary, μ	0.150	0.200	0.250	0.300	0.350	0.400
	Distribution of Progesterone, % by Weight					
0–5	0.2	3.1	7.2	19.0	32.4	47.6
5–10	1.1	5.6	5.9	15.1	23.2	37.4
10–20	10.8	21.9	34.1	65.9	44.4	15.0
20–40	23.3	43.7	52.8	..	..	..
40–60	64.6	25.7	..	..	..	..

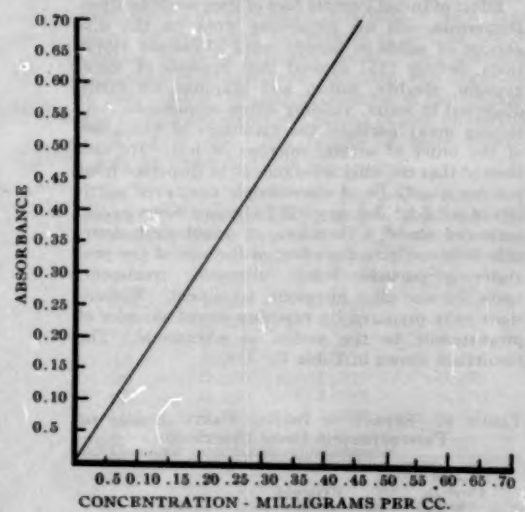

Fig. 1—Relationship between absorbance and concentration of progesterone suspensions.

Thus, the following procedure was adopted for the determination of the effect of ultrasound on the particle size reduction of the suspended progesterone. An aliquot of the suspension was diluted with water to give a concentration of 0.25 mg. of progesterone per cc. which was then transferred to an optically standardized test tube. The absorbance was determined, employing the suspending liquid as a blank having zero absorbance, with a Lumetron[3] photoelectric colorimeter using a 370-mμ filter.

[3] Photovolt Corporation, New York, N. Y.

A melting point determination indicated no decomposition of the progesterone.

Effect of Surface-Active Compounds Upon Dispersion of Progesterone.—Attempts to disperse progesterone in pure distilled water by ultrasonic irradiation gave highly unsatisfactory results because the progesterone was not easily wetted by the liquid.

The addition of surface-active materials has been shown (11) to facilitate the disruption of aggregates of certain materials. A series of suspensions was prepared employing varying concentrations of different surface-active materials as the suspending medium in order to determine the effect of surface-active compounds upon dispersion of progesterone. These results are shown in Tables II and III.

The addition of a surface-active material to the suspending liquid greatly aided the dispersion of the progesterone by ultrasound. The surface-active material allows the liquid to penetrate between the solid surface and the air layers adsorbed on the material causing displacement of the air and resulting in the wetting of the solid.

The lowered interfacial tension at the solid-liquid boundary aids the liquid in penetrating between the individual crystals of the aggregate. This helps in breaking the aggregate into its primary particles. The forces holding the individual particles together are great and energy must be expended in order to overcome these forces. It has been suggested (12) that a surface-active substance might reduce the amount of energy necessary for the disruption of aggregates.

The data in Table II shows that as the concentration of the surface-active material increases to about 0.1–0.2% the dispersion of progesterone also increases. It is possible that at this concentration there is sufficient material present to adequately wet the progesterone particle. As the concentration of surface-active material is increased beyond this

TABLE II.—EFFECT OF CONCENTRATION OF SURFACE-ACTIVE AGENTS UPON DISPERSION OF PROGESTERONE

Concn. of Surface-Active Agent, %	Tween 80	Absorbance Aerosol O.T.	Zephiran Chloride
0.01	0.230	0.100	0.120
0.05	0.250	0.150	0.285
0.10	0.320	0.285	0.310
0.20	0.315	0.335	0.320
0.30	0.310	0.320	0.315
0.40	0.305	0.310	0.305
0.50	0.300	0.300	0.295
1.00	0.295	0.290	0.270
3.00	0.270	0.275	0.250

TABLE III.—EFFECT OF SURFACE-ACTIVE AGENTS UPON DISPERSION OF PROGESTERONE

Surface-Active Agent (0.1%)	Absorbance	Surface-Active Agent (0.1%)	Absorbance
Nonionic		Anionic	
Tween 20	0.330	Aerosol O.T.	0.285
Tween 40	0.320	Ultrawet K	0.340
Tween 60	0.310	Stepanol ME dry AW	0.310
Tween 80	0.315	Stepanol WAT	0.250
G-1020	0.300	Stepanol AM	0.200
Bryj 35	0.330	Stepanol Mg	0.310
Pluronic F-68	0.280		
Superinone P.E.G.[a] 300	0.365	Cationic	
P.E.G. 1500	[b]	Zephiran Chloride	0.310
P.E.G. 4000	[b]	Emulsept	0.310
P.E.G. 9000	[b]	Hyamine 2389	0.150
P.E.G. 600 monolaurate	0.340	Hyamine 10-X	0.330
P.E.G. 1000 monostearate	0.330	Hyamine 1622	0.185
P.E.G. 4000 monostearate	0.295	Bradosol	0.300
P.E.G. 4000 distearate	0.300	Ceepryn	0.270
P.E.G. 6000 monostearate	0.300		
P.E.G. 6000 dilaurate	0.210		

[a] Polyethylene glycol.
[b] Progesterone poorly dispersed; no absorbance measurement taken.

point, the absorbance of the suspension decreases due to the solubilizing action of the surface-active compound. It has been reported by several workers (13, 14) that progesterone can be colloidally dispersed in an aqueous medium by means of high concentrations of surface-active materials.

The differences observed with the various surface-active compounds studied might be attributed to the difference in their wetting ability.

Effect of Concentration of Progesterone upon Dispersion.—The literature contains no information concerning the effect of the concentration of the material being exposed to ultrasonic irradiation upon the resulting particle size. In order to determine the importance of concentration on the particle size of progesterone, a series of progesterone suspensions of varying concentration was prepared. The results of this experiment are shown in Table IV.

TABLE IV.—EFFECT OF CONCENTRATION OF PROGESTERONE UPON DISPERSION

Concentration (mg. per cc.)	Absorbance
5.0	0.360
10.0	0.360
12.5	0.365
25.0	0.362
50.0	0.357
100.0	0.360

As shown in Table IV, the concentration of the material being exposed to ultrasonic irradiation has no effect upon the resulting particle size. Thus, for the preparation of suspensions by ultrasonic energy it would be more economical to irradiate a concentrate of the material to be dispersed which could then be diluted to produce a suspension of the desired concentration.

Effect of Initial Particle Size of Progesterone Upon Dispersion.—In his pioneering work on the dispersion of solids in liquids using ultrasonic vibrations, Sollner (15) showed that crystals of mica, gypsum, steatite, sulfur, and graphite are easily dispersed in water, yielding dilute suspensions containing many particles the diameters of which are of the order of several microns or less. He also showed that the solid substance to be dispersed need not necessarily be of macroscopic size; even particles of colloidal size may still be broken down by the action of sound. Therefore, it would seem desirable to investigate the effect of the size of the progesterone particles before ultrasonic treatment, upon the size after ultrasonic treatment. Suspensions were prepared by exposing sieved samples of progesterone to the action of ultrasound. The results are shown in Table V.

TABLE V.—EFFECT OF INITIAL PARTICLE SIZE OF PROGESTERONE UPON DISPERSION

Passed Through Sieve Number	Retained on Sieve Number	Absorbance
40	60	0.260
60	80	0.283
80	100	0.290
100	120	0.312
120	200	0.340
200	—	0.360

From the results shown in Table V, it can be seen that the initial particle size does influence the particle size of the progesterone after a standard ultrasonic exposure. That is, the smaller the initial size of the particles, the smaller will be the size of the suspended particles after ultrasonic irradiation for a standard period of time. However, the same size may be obtained if the sample originally containing the larger particles is irradiated for a longer period

of time. For example, a sample of 200-mesh progesterone yielded a suspension with an absorbance of 0.360 after a fifteen-minute exposure. After the same period of irradiation 80-mesh progesterone produced a suspension with an absorbance of 0.290. However, continued exposure of this sample for another fifteen-minute period produced a suspension with an absorbance of 0.360.

Effect of Time of Ultrasonic Irradiation Upon Dispersion of Progesterone.—In studying the effects of ultrasonic energy upon dispersion it has been found that there is an optimum time for ultrasonic irradiation to produce maximum dispersion. For the dispersion of barium sulfate particles, Matieu-Sicaud and Levavasseur (16) have shown that maximum dispersion was obtained after irradiation for twenty minutes, and no advantage was obtained by irradiation for longer periods of time. Increasing the time of insonation of clay suspensions beyond ten minutes and up to two hours had no effect upon the results obtained (17). Employing ultrasonics for the dispersion of a dye it was found (2) that during the first five to ten minutes a rapid breaking up of the particles takes place. Continued insonation does not reduce the size of the dye particles any further.

In order to study the effect of time of ultrasonic exposure upon dispersion, suspensions were prepared by insonation for periods of five to thirty minutes at three intensity levels. One hundred milligrams of progesterone, 200-mesh, was dispersed in 10 cc. of a 0.1% aqueous solution of Superinone. The exposure chamber was placed 34 mm. above the vibrating crystal. The results of these experiments are shown in Table VI.

TABLE VI.—EFFECT OF TIME OF ULTRASONIC IRRADIATION UPON DISPERSION OF PROGESTERONE

Exposure Time (min.)	Absorbance		
	17.5 watts[a]	42.5 watts[a]	70.0 watts[a]
5	0.175	0.285	0.310
10	0.230	0.300	0.340
15	0.260	0.320	0.370
20	0.260	0.325	0.370
25	0.260	0.325	0.370
30	0.260	0.325	0.370

[a] Ultrasonic intensity in sound chamber.

As shown in Table VI, maximum dispersion is reached by insonation for a period of fifteen to twenty minutes. Ultrasonic irradiation for periods up to thirty minutes produced no measurable effect.

For each ultrasonic intensity the maximum dispersion is attained after insonation for approximately the same period of time. However, the extent of dispersion is directly related to the ultrasonic intensity applied.

Effect of Ultrasonic Intensity Upon Dispersion of Progesterone.—When considering the effect of ultrasonic energy upon suspensions, the literature has already indicated the importance of the intensity of these vibrations. Several workers have shown that ultrasound can affect suspended material in one of two diametrically opposed manners, depending upon the intensity of the vibrations.

In studying the effect of ultrasonic vibrations upon clay suspensions it has been found (17) that mild intensity ultrasound, below cavitation level, increased the coagulation of the dispersed material. However, high intensity insonation, above the cavitation threshold, caused pronounced dispersion of the clay agglomerates. Sollner and Bondy (18) have also shown that an energy exceeding a certain limit is needed for dispersion and that below this limit coagulation will predominate.

In order to study the effect of ultrasonic intensity upon the dispersion of progesterone, suspensions were prepared at different ultrasonic intensities. The acoustical intensity was varied by changing the electrical potential impressed on the crystal. Suspensions were prepared employing 100 mg. of 200-mesh progesterone in 10 cc. of a 0.1% aqueous solution of Superinone. The samples were exposed to ultrasonic vibrations for a fifteen-minute period with the exposure chamber placed 34 mm. above the vibrating crystal. The results of this study are shown in Table VII.

TABLE VII.—EFFECT OF ULTRASONIC INTENSITY UPON DISPERSION OF PROGESTERONE

Ultrasonic Intensity (ma.)[a]	Ultrasonic Intensity (watts)[b]	Absorbance		
		300 kc.	500 kc.	750 kc.
100	17.5	0.260	0.250	0.255
125	30.0	0.295	0.290	0.295
150	42.5	0.320	0.315	0.320
175	57.5	0.355	[c]	[c]
200	70.0	0.370	[c]	[c]

[a] Acoustical intensity as measured by the oscillator plate current in milliamperes.
[b] Acoustical intensity in sound chamber as measured calorimetrically.
[c] These intensity levels could not be attained with apparatus available.

It can be seen from Table VII that dispersion increases as the ultrasonic intensity is increased. It has been reported (19) that there is an optimum intensity for production of cavitation which coincides with the maximum formation of cavitation bubbles. At ultrasonic intensities above this optimum there is a decrease in the effects produced by cavitation. However, such a decrease in cavitation effects was not attained in this investigation.

SUMMARY

1. The suitability of employing ultrasound for the preparation of pharmaceutical suspensions was investigated.

2. A turbidimetric method was devised for determining the efficiency of the ultrasound in reducing the particle size of the suspended material.

3. The addition of a surface-active material to the suspending liquid was found necessary to produce a satisfactory dispersion.

4. The concentration of the dispersed material had no effect on the resulting particle size.

5. The final particle size was directly proportional to the initial particle size, after a standard ultrasonic exposure.

6. Maximum dispersion was attained by

insonation for a period of fifteen to twenty minutes when 200-mesh progesterone was employed. The use of progesterone of a larger initial size increased the time required to produce maximum dispersion. Once maximum dispersion was attained insonation for longer periods produced no measurable effect.

7. Dispersion increased as the ultrasonic intensity was increased.

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A Study of Dispersion with Ultrasound II*

By BERNARD MISEK† and DONALD M. SKAUEN‡

The importance of several factors influencing dispersion with ultrasound is discussed. It was found that the extent of dispersion decreased as the viscosity of the suspending liquid increased. At lower temperatures, the temperature of the suspending liquid had no effect upon dispersion. However, near the boiling point of the suspending liquid there was a considerable decrease in the extent of dispersion. The application of a 15-lb. pressure upon the suspending liquid during ultrasonic exposure rendered dispersion most efficient. Dispersions so produced contained 85 per cent, by weight, of the suspended material as particles of 10 microns or less in size. The various steroids studied differed in their ease of dispersion. An increase in dispersion occurred when the distance between the under surface of the exposure chamber and the upper surface of the oscillating crystal was a whole number of half-wavelengths. At the frequencies studied, the frequency of the ultrasonic vibrations had no effect upon dispersion.

SOME OF THE RESULTS obtained in the study of dispersion with ultrasonic vibrations were reported in a previous paper (1). In the present investigation several of the factors which influence dispersion with ultrasound are discussed. This includes the importance of the viscosity and temperature of the suspending liquid, the pressure exerted upon the liquid, the relative ease of dispersion of the various steroids studied, the effect of the orientation of the exposure chamber, and the frequency of the ultrasonic vibrations.

The ultrasonic generator, stainless steel exposure chamber, and the turbidimetric method used to evaluate the effect of ultrasonic energy on dispersion described in the previous paper (1) were used in this investigation.

Except where otherwise indicated, all samples were exposed to ultrasonic irradiation for fifteen minutes at a frequency of 300 kc. per second. The oscillator plate power was set at

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175 ma. producing approximately 57.5 watts of acoustical energy in the sound chamber, as determined calorimetrically. The distance between the base of the exposure chamber and the top of the vibrating crystal was 34 mm. Each sample contained 100 mg. of progesterone, 200-mesh, dispersed in 10 cc. of a 0.1% aqueous Superinone[1] solution.

EXPERIMENTAL

Effect of Viscosity of the Suspending Liquid Upon Dispersion of Progesterone.—Several workers (2, 3) have indicated the importance of viscosity in emulsification by ultrasound. They have shown that the highest degree of dispersion is obtained with oils having the lowest viscosity.

A series of suspensions was prepared employing, in each sample, 0.5% of either methylcellulose or carboxymethylcellulose in addition to the 0.1% Superinone. The results of these experiments are shown in Table I.

TABLE I.—EFFECT OF VISCOSITY OF THE SUSPENDING LIQUID UPON DISPERSION OF PROGESTERONE

Suspending Liquid[a]	Absorbance
Control (no gum)	0.365
Carboxymethylcellulose	
Low Viscosity	0.340
Medium Viscosity	0.320
High Viscosity	0.280
Methylcellulose 100 c. p. s.	0.330
400 c. p. s.	0.310
1500 c. p. s.	0.290
4000 c. p. s.	0.270

[a] 0.5% gum was dissolved in a 0.1% Superinone solution.

It can be seen from Table I that in suspensions prepared by ultrasonic energy the dispersion of progesterone decreases as the viscosity of the suspending liquid is increased. At a constant acoustical intensity, the extent of cavitation[2] in a liquid decreases as the viscosity of the liquid is increased. Since the mechanical effects of ultrasonic irradiation result from the collapsing cavities (4) any decrease in the extent of cavitation would manifest itself in a decrease in the effects produced by this phenomenon, as was shown above. It follows, therefore, that if a material is to be added to a suspension for the purpose of increasing the viscosity, it would be advisable to do so after ultrasonic irradiation in order to obtain maximum dispersion under a given set of conditions.

Effect of Temperature of the Suspending Liquid Upon Dispersion of Progesterone.—When a liquid is exposed to ultrasonic irradiation, there is a marked rise in the temperature due to the partial conversion of the sound energy to heat. These thermal effects are greatly increased in the presence of extended phase boundaries as they occur in micro-heterogeneous systems, such as suspensions (5). It has been reported that the effects of cavitation vary with the temperature of the irradiated liquid (6). Metal surfaces exposed to sound energy have been found to exhibit a maximum of cavitation erosion at about 60°. At higher temperatures the extent of cavitation erosion decreases with increasing temperatures.

In order to study the effect of temperature upon dispersion by ultrasound, suspensions were prepared with the suspending liquid at various temperatures. Temperature control was obtained by means of water circulating through a coil placed in the sound bath. The results of this study are shown in Table II.

TABLE II.—EFFECT OF TEMPERATURE OF SUSPENDING LIQUID UPON DISPERSION OF PROGESTERONE

Temp. of Irradiated Liquid (°C.)	Absorbance
10	0.365
25	0.365
50	0.365
95	0.240

From Table II it can be seen that at the lower temperatures, the temperature of the suspending liquid has no effect upon dispersion produced by ultrasound. However, near the boiling point there is a considerable decrease in the extent of dispersion produced because the cavities which are formed in the liquid contain much vapor due to the high vapor pressure of the water. When the cavity collapses, this vapor acts as a buffer and thus decreases the vehemence of the collapse (7). As a result, the collapse is not as powerful and the mechanical effects are not as great as at the lower temperatures when there is less water vapor present in the cavity. Therefore, during extended periods of insonation, when the temperature of the suspending liquid may rise to a high level it is advisable to provide some means for the removal of this excess heat.

Effect of Pressure On The Suspending Liquid Upon Dispersion of Progesterone.—Sollner and Bondy (7) have indicated that a certain external pressure upon the liquids being emulsified renders emulsification by ultrasonic irradiation most efficient. They showed that the percentage of the dispersed phase increases with the pressure upon the liquids until a pressure of 1500 mm. of mercury is reached. Increasing the pressure beyond this value causes the amount of the dispersed phase to decrease sharply.

With these facts in mind, it was decided to study the effect of applied pressure on the suspending liquid upon the dispersion of progesterone produced by ultrasound. The exposure chamber employed previously in this investigation could not be used in this part since its diaphragm would not withstand the applied pressure. A Pyrex test tube having an inside diameter of 25 mm. and a length of 200 mm., was utilized for exposing samples to the ultrasonic irradiation. Air pressure was maintained by means of an air compressor connected to the test tube. Cylinders of nitrogen and carbon dioxide were employed to maintain the pressure of these gases. The results of these experiments are shown in Table III.

It should be pointed out that the glass walls of the test tube do not allow the maximum transmission

[1] Winthrop Laboratories, Inc., New York, N. Y.
[2] The formation and subsequent collapse of cavities produced in a liquid during ultrasonic irradiation.

TABLE III.—EFFECT OF PRESSURE ON THE SUSPENDING LIQUID UPON DISPERSION OF PROGESTERONE

Applied Pressure (lb.)		Absorbance: Carbon Dioxide	Nitrogen	Air
7.5 lb. Vacuum		...	...	0.240
Atm. pressure		...	...	0.310
Applied pressure	5.0	0.350	0.310	0.330
	10.0	0.350	0.370	0.350
	15.0	0.360	0.400	0.410
	20.0	0.350	0.390	0.410
	25.0	0.340	0.380	0.400
	30.0	0.340	0.380	0.400

of ultrasonic energy into the vessel. Hence, it will be noted that at atmospheric pressure an absorbance of 0.310 is obtained while under the same conditions, employing the exposure chamber with the acoustically transparent bottom, an absorbance of 0.365 is obtained.

Thus, it is evident from Table III, that at an applied pressure of 15 lb. dispersion is rendered most efficient. Dispersions so produced contained 85%, by weight, of the suspended material as particles of 10μ or less in size. This may be explained by the fact that during cavitation, the cavities produced in the liquid collapse with different vehemence depending upon the applied pressure. At low pressures, the cavities are formed, but they do not collapse because the vapor pressure within the cavities is practically in equilibrium with the outside pressure. At higher pressures, the formation of cavities is rendered more difficult because of the fact that the energy of ultrasonics can overcome only a limited applied pressure when forming cavities (7).

The differences observed when employing the various gases can be explained from the standpoint of the solubility of the gas in water. Dissolved gases favor the formation of nuclei and thus promote the formation of cavities. However, at higher concentrations, as would be obtained at the higher gas pressures, these gases have a buffering effect upon the collapse of the cavities. Therefore, when the relatively insoluble gases, such as air and nitrogen, are employed, they produce a pressure upon the surface of the liquid, without dissolving in the liquid to any great extent. The carbon dioxide, being more soluble, dissolves to a greater extent than the other gases and buffers the collapse of the cavities.

Effect of Ultrasound Upon Dispersion of Various Steroids.—Microscopic observation of many of the steroids show them to exist as aggregates of numerous individual particles. It is generally agreed (8, 9) that the primary purpose of most dispersion procedures is the separation of the aggregate into its individual primary particles. The actual breakdown of the individual primary particles, however, is highly unlikely when using the conventional dispersion methods.

The primary particles are formed by the condensation of ions or molecules. These individual particles then unite to form the aggregate. The forces holding the individual particles in the form of the aggregate are usually electrostatic in nature. They are due to the unbalanced surface charges on the particles and will vary with the electronic configuration of the molecule of the material in question. Thus, the energy needed to separate these particles will vary with the forces holding them together. It is, therefore, quite conceivable that, under the same conditions, one material will be adequately dispersed to produce a good suspension while another material will yield a poor dispersion.

The following experiment was designed to study the dispersion of various steroids by ultrasonic irradiation. A sample of each steroid was passed through a 200-mesh screen and was dispersed by manual shaking in the suspending vehicle. The absorbance of the suspension was then determined. A similar sample was exposed to ultrasonic irradiation for fifteen minutes and its absorbance determined. The results of these experiments are shown in Table IV.

TABLE IV.—EFFECT OF ULTRASOUND UPON DISPERSION OF VARIOUS STEROIDS

Steroid	Absorbance: Before Irradiation	After Irradiation
Progesterone	0.150	0.365
Estrone	0.105	0.270
Estradiol benzoate	0.230	0.390
Testosterone	0.125	0.510
Testosterone ethoxyacetate	0.060	0.230

It can be seen from Table IV that materials vary widely in their ease of dispersion; that is, under the same conditions, one material breaks up only slightly producing a coarse suspension, whereas the aggregates of another material may be greatly dispersed and yield a fine suspension.

Effect of Orientation of Exposure Chamber upon Dispersion of Progesterone.—Several investigators (10, 11) have indicated that the location of the exposure chamber in relation to a vibrating flat-faced crystal will influence the ultrasonic intensity in the chamber.

TABLE V.—EFFECT OF ORIENTATION OF EXPOSURE CHAMBER UPON DISPERSION OF PROGESTERONE

Distance Above Crystal (mm.)	Absorbance
34.0	0.360
33.5	0.345
33.0	0.325
32.5	0.320
32.0	0.340
31.5	0.355
31.0	0.340
30.5	0.330
30.0	0.340
29.5	0.345
29.0	0.353
28.5	0.350
28.0	0.340
27.5	0.335
27.0	0.335
26.5	0.350
26.0	0.360
25.5	0.347
25.0	0.330
24.5	0.337
24.0	0.359
23.5	0.340
23.0	0.330
22.5	0.330
22.0	0.337

In order to study the effect of orientation of the exposure chamber upon dispersion, a micrometer screw was attached to the clamp holding the exposure chamber. Starting with a distance of 34.0 mm. between the diaphragm of the chamber and the flat-faced crystal, the container was lowered 0.5 mm. in each step and suspensions prepared at each setting. The results of these experiments are shown in Table V.

It can be seen from Table V that at certain distances between the vibrating crystal and the exposure chamber dispersion is at a maximum.

Since the velocity of sound waves in water at 25° is 1457 meters per second, at 300 kc. per second the wavelength would be 4.86 mm. and a half-wavelength would be 2.43 mm. Maximum particle size reduction occurs at points approximately a half-wavelength apart in the lowering of the exposure chamber toward the crystal. Under these conditions the wave which strikes the bottom of the exposure chamber is reflected back towards the vibrating crystal when its phase is such as to reflect the wave back towards the vessel. The sound wave is thus imprisoned by multiple reflections between the vibrating crystal and the underside of the exposure chamber and the energy entering the exposure chamber rises to a high value. At other distances the reflected wave is out of phase with the oscillating crystal and the stationary wave pattern does not result (3).

Effect of Frequency of Ultrasonic Vibrations Upon Dispersion of Progesterone.—There is conflicting information in the literature concerning the importance of ultrasonic frequency upon dispersion. Thompson and Vilbrandt (12) have concluded that the frequency of insonation had no effect upon the dispersion or coagulation of clay suspensions. However, it has also been reported (13) in a study of dispersion as a function of ultrasonic frequency, that dispersion increases to a maximum at 1000 kc. and then decreased with increased frequency.

The following experiment was designed in order to study the importance of the frequency of the ultrasonic vibrations upon the dispersion of progesterone. The suspensions were prepared employing three frequencies of insonation, 300, 500, and 750 kc. per second. The results of this study are shown in Table VI.

TABLE VI.—EFFECT OF FREQUENCY OF ULTRASONIC VIBRATIONS UPON DISPERSION OF PROGESTERONE

Frequency (kc. per sec.)	Absorbance
300	0.320
500	0.315
750	0.320

From Table VI it is evident that at the levels studied, frequency has no effect upon dispersion of the progesterone.

SUMMARY

1. The importance of several factors influencing dispersion with ultrasound is discussed.

2. The extent of dispersion decreased as the viscosity of the suspending liquid increased.

3. At the lower temperatures, the temperature of the suspending liquid had no effect upon dispersion. However, near the boiling point of the suspending liquid there was a considerable decrease in the extent of dispersion.

4. Dispersions produced with the application of 15 lb. pressure upon the suspending liquid during ultrasonic exposure contained 85%, by weight, of the suspended materials as particles of 10 microns or less in size.

5. The various steroids studied differed in their ease of dispersion.

6. An increase in dispersion occurred when the distance between the under surface of the exposure chamber and the upper surface of the oscillating crystal was a whole number of half-wavelengths.

7. At the frequencies studied, the frequency of the ultrasonic vibrations had no effect upon dispersion.

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The Calibration and Use of Direct Reading Isotonicity Meters*

By BERTON E. BALLARD and FRANK M. GOYAN

A thermistor thermometer for determining freezing-point depressions is described in principle. Methods for establishing scales for the direct reading of freezing-point depressions and the corresponding isotonicity values in per cent NaCl are discussed in detail. It is shown that a linear isotonicity scale defined in terms of per cent NaCl is practicable. The instrument is easily calibrated by taking advantage of two fixed temperatures, one established by the ice point and the other by a three-phase equilibrium made by partially freezing one selected saturated solution. Several saturated solutions suitable for this purpose have been studied and their freezing points and corresponding isotonicity scale values are reported.

THE CONVENIENCE of calculating osmotic properties of solutions by assuming the additivity of osmotic equivalent amounts of NaCl has been recognized in pharmacy for many years. Several years ago one of the authors, Goyan (1), conceived the idea of developing a direct reading isotonicity meter based upon this method of calculation. It can be shown that the errors involved by assuming the additivity principle are less than those of acceptable routing experimental measurements. This discovery has led to a defined isotonicity scale which can be applied to any thermometer suitable for measuring freezing-point depressions.

The development of the linear scale thermistor thermometer (2) makes it entirely practical to design a direct reading isotonicity meter if a linear isotonicity scale can be agreed upon and if suitable methods for calibration are available. The methods described below accomplish this purpose for freezing-point measurements although the concept may be applied to any instrument capable of indicating some colligative property of a solution on a linear scale.

In this paper the physicochemical usage of the term isotonicity as recommended by Goyan and Reck (1) is used. This usage implies the comparison of osmotic pressure through ideal semipermeable membranes or comparison by the determination of other colligative properties such as the lowering of the freezing point. There is little hope of developing a direct reading instrument based upon osmotic pressure across specific animal membranes. In cases where this effect is different from that produced by the ideal membrane, the exact nature of the membrane must be specified in every case.

* Received May 3, 1957, from the University of California School of Pharmacy, San Francisco 22.
Presented to the Scientific Section of the A. PH. A., New York meeting, April 1957.

EXPERIMENTAL

Isotonicity Scale.—Based upon the concept that two solutions are isotonic if they have the same freezing point, a linear isotonicity scale is defined by the established value of 0.53° for the freezing-point depression of a 0.90% solution of NaCl (3, 4). This statement may be reduced to the equation.

$$I\rho = (0.90/0.53)\,\Delta T \qquad \text{(Eq. 1)}$$

$I\rho$ represents the isotonicity value of a solution in per cent (w/v) of NaCl at 25° and ΔT stands for the corresponding freezing-point depression in ° C. Solutions that cannot be studied by the freezing-point method because of the precipitation of solute on cooling, or for other reasons, require special consideration. Any other colligative property may be substituted for ΔT if the corresponding value of this property for a 0.90% solution of NaCl is substituted for the factor 0.53 in Eq. 1.

The scale defined by Eq. 1 is shown in Fig. 1. It will be noted that the isotonicity scale is arbitrarily defined in such a way as to constitute a new system of units for measuring freezing-point depression.

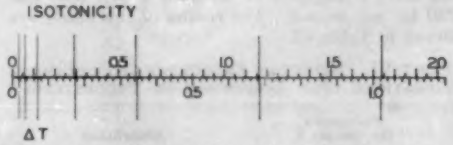

Fig. 1.—The isotonicity scale (% NaCl) is shown above a scale showing freezing-point lowering (°C). *Long vertical lines* extending up from the isotonicity scale mark points calculated from concentration data of Scatchard and Prentiss for NaCl. *Long vertical lines* extending down mark corresponding measured freezing-point depressions.

Justification for this procedure is based on the fact that isotonicity scale readings established by freezing-point measurements of pure NaCl solutions agree within 0.01% NaCl with corresponding positions calculated from concentrations over the 2% range displayed. The long lines shown in Fig. 1 represent data for NaCl given by Scatchard and Prentiss (5). The lines intersecting the ΔT scale represent values obtained from measurements of

the freezing-point depression for NaCl solutions. Similar lines on the isotonicity scale mark positions calculated from the published molality of these solutions. Transformations to percentage values were made with the aid of functions of density taken from Harned and Owen for 25° (6). A maximum difference of 0.007% NaCl is observed at the scale position of approximately 0.5% NaCl. This difference drops off gradually to 0.000 in very dilute solutions and changes sign at about 1.2%. At 1.7% the isotonicity value calculated from freezing-point data is less than the actual percentage by 0.005%. This difference reaches 0.011 at 2.3%, 0.014 at 2.9%, and drops back to 0.011 at 4%.

It will be noted that the deviations up to 2% NaCl are well within the limits of error of most routine measurements. It is not recommended that freezing-point measurements of high precision be stated in *Ip* units. However, no actual error will result by following this practice since *Ip* units are defined in terms of freezing-point lowering.

Instrumentation.—A differential mercury thermometer, a calorimeter-type thermometer, and a thermistor thermometer were used in this development.

Various circuit modifications of the thermistor thermometer have been used. The circuits are based upon measuring the voltage produced across a slightly unbalanced thermistor bridge by means of a balanced triode vacuum tube voltmeter, using one 6 SN 7 GT tube with plate voltage stabilized with an OA3 regulator. Sensitivity is controlled by means of a variable resistor in series with a 0–100 microammeter. Sensitivity is made high enough so that the full scale may correspond to less than 1°. Early modifications were designed with a dual meter scale patterned after the one shown in Fig. 1. At present, conventional methods are used to switch from a freezing-point depression scale to an isotonicity scale using the conversion factor of Eq. 1 in choosing voltage-range resistors.

The thermistors used were type 51A1 made by Victory Engineering Corporation of Union, N. J. These thermistors consist of sensitive elements sealed in the end of short, glass rods 2–3 mm. in diameter. The freezing-point technique used requires a sturdy probe about the length of an ordinary thermometer. This probe is prepared by sealing one end of a Pyrex tube about 25 cm. x 6 mm. i. d. A slight constriction is made 3–4 cm. from the sealed end in order to produce a bulb which will serve to hold the sensitive element in contact with mercury.

The leads of one of these thermistors are soldered to a length of fairly stiff plastic-coated microphone cable and one lead is insulated with sleeving. The insulation is completed with Scotch electrical tape which is also used to secure a wad of glass wool in such a position that it will serve as a plug to hold the mercury in the bulb. The final assembly is accomplished by pouring mercury into the bulb and inserting the thermistor and its cable down the tube until the sensitive element is well below the surface of the mercury and the glass wool becomes an effective plug which prevents the mercury from leaving the bulb. The microphone cable is stiff enough to hold the plug under tension after the cable has been secured near the open end of the tube.

Calibration and Use.—The thermistor thermometer requires an occasional adjustment of sensitivity as well as the usual adjustment for the ice point. Any suitable concentration of NaCl would serve this purpose, but is not entirely satisfactory for calibration because its exact freezing point can be read only when the amount of ice in equilibrium with the original solution is small. Rather than employ methods requiring analysis after equilibrium has been attained, three-phase systems were sought which hold a constant freezing temperature during the melting of most of the ice present. Several saturated solutions were selected for study on the basis of solubility at 0°, molecular weight and molal freezing-point depression.

Saturated solutions of the compounds were made at room temperature and their freezing points determined as follows: Large test tubes (25 x 200 mm.) were selected so as to fit loosely into the neck of a narrow-neck, quart vacuum bottle. A rubber ring cut from rubber tubing was stretched over each tube just below the lip, in order to minimize the danger of accidental breakage when the tubes were hung by the lip in the vacuum bottle. The tubes were selected so as to allow space for a small amount of ice in the bottom of the vacuum bottle without bringing the lower parts of the tubes into contact with anything but cold air contained in the bottle.

A determination is made by partly filling a tube with saturated solution and freezing the contents by immersing in an ice-salt freezing mixture with intermittent stirring until a considerable amount of ice and precipitated solute forms. The tube and contents are then transferred to the vacuum bottle and stirred with a loop of stainless steel wire shaped so as to let the lower end of the wire scrape the side of the tube. The temperature is observed with thermometers. Results obtained with a calorimeter-type thermometer (Brooklyn Thermometer Co. #F3950, −6° to +6° in 0.01°) and with a differential thermometer of the Philadelphia type (both corrected for the ice point) agreed within 0.01°. No attempt was made to purify the reagent-quality compounds used. Selected results are shown in Table I. Saturated solutions of mercuric chloride (f. p. = −0.28) and of chlorobutanol (f. p. = −0.07) were measured but not adopted for standardization purposes; the former because of reaction with metal stirrers and the latter because of the low value obtained.

TABLE I.—SATURATED SOLUTIONS FOR STANDARDIZING DIRECT READING ISOTONICITY METERS

Substance	Freezing Point Scale Reading, °C.	Isotonicity Scale Reading, % NaCl
KIO_3	0.68	1.15
H_3BO_3	0.77	1.31
$KClO_3$	0.82	1.39

Freezing-point measurements of ordinary solutions are made in the same way except that the tube is removed from the cold air bath in the vacuum bottle from time to time for inspection and for the purpose of melting excessive ice. After inspection, the tube is returned to the cold air and a new freezing point taken. Violent stirring with the stainless

steel wire loop is required. This process of inspection has the distinct advantage that with very little experience it is possible to obtain reproducible results which agree with known concentrations of NaCl within 0.02% without plotting cooling curves or removing samples for analysis. Furthermore, frequent inspection gives a quick and sure test of the integrity of the results with respect to possible precipitation of solute as well as to the possible increase in concentration due to excessive ice formation.

DISCUSSION

There is good evidence that the thermistor thermometer is the instrument of choice for this work. Several modifications of the instrument have been used by classes of students who seem to prefer it to ordinary thermometers because of the ease of reading the scale. The method of quick freezing and subsequent inspection is greatly facilitated by a thermistor probe which allows freedom to remove the freezing tube from the air bath while maintaining a permanently located reading scale.

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Cyanocobalamin (Vitamin B_{12}) II.

Further Studies of the Effect of Ascorbic Acid Degradation Products on Cyanocobalamin*

By A. J. BARTILUCCI, R. DiGIROLAMO, and H. EISEN

The data presented seem to indicate that the stability of cyanocobalamin is affected by dehydroascorbic acid, a decomposition product of ascorbic acid. This study further corroborates the initial findings of the effect of decomposed ascorbic acid on cyanocobalamin.

IN A PREVIOUS PUBLICATION (1) it was suggested that the decomposition products of ascorbic acid are significantly involved in the decomposition of cyanocobalamin in aqueous solution.

Further investigations are reported indicating the effect of varying concentrations of ascorbic acid decomposition products on cyanocobalamin; and the effect of dehydroascorbic acid, the reversible oxidative product encountered in the stepwise degradation of ascorbic acid, on cyanocobalamin.

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Project financially supported by Charles Pfizer & Co., Inc., Brooklyn, N. Y.

EXPERIMENTAL AND RESULTS

Ascorbic acid was determined by titration in a 1% oxalic acid solution with 0.1 *N* iodine, using starch T. S. as the indicator. The cyanocobalamin was assayed by a procedure described in the Difco Manual (2) using *L. leichmannii* 4797 ATCC as the test organism. The possible effect of the decomposition products of ascorbic acid on the microbial assay was not determined. Generally, determinations were discontinued when 50 to 75% decomposition had taken place.

The cyanocobalamin used was in the form of a 0.1% trituration with mannitol as the diluent. The mannitol in the concentration present did not appear to affect the results. The ascorbic acid used in the study was of U. S. P. quality in a fine crystalline form. The water used as a solvent was distilled water redistilled from an all-glass still. The dehydroascorbic acid was obtained from the

TABLE I.—PERCENTAGE LOSS OF CYANOCOBALAMIN IN ASCORBIC ACID SOLUTION AND DECOMPOSED ASCORBIC ACID SOLUTIONS AT 40°

	A.A.	B_{12}	A.A.	B_{12}	A.A.	B_{12}	A.A.	B_{12}
Initial	0.0	0.0	9.2	0.0	23.5	0.0	68.1	ca 60
1 week	19.4	10.6	15.5	36.7	31.4	52.8	..	..
2 weeks	25.3	50.6	19.2	78.7	34.8	100.0	..	..
1 day	..	..	..	..	..	..	68.8	67.3
2 days	..	..	..	..	..	..	68.7	79.5

TABLE II.—PERCENTAGE LOSS OF CYANOCOBALAMIN IN SOLUTIONS OF DEHYDROASCORBIC ACID AT 40°

	—Dehydroascorbic Acid, mg./ml.—		
	5	12.5	25
Initial	5	24	44
1 day	15	40	60
2 days	26	54	72
3 days	36	70	..
4 days	46	79	..
5 days	54	..	..
6 days	64	..	..
7 days	76	..	..

National Biochemicals Co., Cleveland, Ohio, and decomposed at 225°. Containers, pipets, and all glassware used were thoroughly washed, rinsed several times with glass-distilled water, and air dried before use.

Five-hundredths per cent of propylparaben was included in all samples as an antimicrobial preservative. The solutions were subdivided into one-ounce flint glass, screw-capped bottles and stored in cardboard boxes at 40°.

The stability data of a solution initially containing 50.0 mg./ml. of ascorbic acid and 11.0 mcg./ml. of cyanocobalamin stored at 40° appear in the first two columns of Table I.

The effect of varying concentrations of ascorbic acid decomposition products on the stability of cyanocobalamin was then investigated.

A stock solution of ascorbic acid containing 51 mg./ml. as determined by assay, was stored at 40°. Progressive decomposition was followed by periodically removing aliquot portions and determining the ascorbic acid content remaining therein. Three test solutions indicating 9.2, 23.5, and 68.1% decomposition of ascorbic acid were removed.

An amount of cyanocobalamin was added to these solutions which would be expected to yield a solution containing 10 mcg./ml. Initial assays indicated a cyanocobalamin content in the first two test solutions of 10.7 and 11.0 mcg./ml., respectively. In the test solution containing the 68.1% decomposed ascorbic acid, the initial microbial assay indicated a cyanocobalamin content of 4.0 mcg./ml.

All solutions were stored at 40° and the ascorbic acid and cyanocobalamin content determined periodically. The first two solutions were assayed at weekly intervals. The third solution was assayed daily. The results appearing in Table I indicate a progressive loss of cyanocobalamin in direct ratio to the degree of ascorbic acid decomposition.

On the basis of the change in color observations previously reported (1) and the experiments reported above, an investigation of the effect of one of the known decomposition products of ascorbic acid on cyanocobalamin seemed warranted. Dehydroascorbic acid is the initial oxidation product of ascorbic acid. While this reaction is reversible, further decomposition of dehydroascorbic acid eventually leads to extensive degradation with the formation of oxalic acid and 2,3-diketogulonic acid.

Accordingly, then, each of three solutions was prepared to contain 10 mcg./ml. of cyanocobalamin and 5 mg., 12.5 mg., and 25 mg./ml. of dehydroascorbic acid, respectively. The quantity of dehydroascorbic acid used was calculated to be equivalent to the amount present in solutions containing respectively 10, 25, and 50% decomposed ascorbic acid based upon the assumption that dehydroascorbic acid was the only decomposition product present. Initial microbial assays indicated from possibly slight to marked destruction of the cyanocobalamin in the solutions.

The solutions were stored at 40° and the cyanocobalamin content determined daily. The assay data summarized in Table II indicate that the rate of decomposition of cyanocobalamin increases as the initial concentration of dehydroascorbic acid increases.

SUMMARY

The stability of cyanocobalamin was shown to be decreased in the presence of decomposed ascorbic acid. Dehydroascorbic acid, the first decomposition product of ascorbic acid, caused greater instability of cyanocobalamin in solution than ascorbic acid. Investigations are continuing on the effect of other known decomposition products of ascorbic acid on cyanocobalamin, and it is expected that the results will be published.

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The Effect of Certain Additives on the Gel Point of Methylcellulose*

By GERHARD LEVY and T. W. SCHWARZ

The effect of certain additives on the gel point of various grades of Methocel 4000 solutions has been investigated. Most electrolytes, as well as sorbitol, sucrose, and glycerin depress the gel point while ethanol, propylene glycol, and polyethylene glycol 400 raise the gel point. The effect of propylene glycol in counteracting the gel point depression caused by magnesium chloride or sucrose as examples of electrolytes and nonionic hydrophilic compounds, respectively, is illustrated. The gel point raising effect of ethanol, propylene glycol, and polyethylene glycol 400 has been related to their solvent properties.

METHYLCELLULOSE,[1] a methyl ether of cellulose, is a hydrophilic colloid which is used in pharmacy to increase the viscosity of many aqueous preparations.

The polymer is soluble in cold water, but insoluble in hot water. When methylcellulose solutions are heated, they become cloudy and gel, as evidenced by an abrupt increase in the viscosity of such solutions. The temperature, at which gelling occurs is referred to as the "gel point." The gel point is dependent on the concentration, viscosity type, and grade of methylcellulose as well as on the presence and the concentration of certain other compounds in the solution.

The term "viscosity type" refers to the degree of polymerization of the gum, and is expressed in terms of the approximate viscosity of a 2 per cent solution. Designations for Methocel types range from 10 to 7,000, that is, their 2 per cent aqueous solutions have a viscosity range of 10 to 7,000 c. p. s. under standard conditions.

In general, the higher the viscosity of a methylcellulose solution, the lower is the temperature at which gelling will occur. The addition of most electrolytes causes a lowering of the gel point. The mechanism of the sol-gel transformation of methylcellulose has been studied by Heymann (1) who reports that the gel point depression by electrolytes is primarily related to the concentration and kind of anions present. The lyotropic series is valid in that thiocyanate and iodide raise the gel point, while all other anions in the series will lower the gel point.

Pharmaceutical preparations containing methylcellulose in relatively high concentration, such as lubricant and contraceptive jellies, and water-soluble ointments, or preparations containing methylcellulose together with electrolytes or certain other compounds, will frequently form a stiff and opaque gel at room temperature or at slightly elevated temperatures. Such a sol-gel transformation results in a product which cannot be extruded from a collapsible tube or poured from a bottle.

Recently, a number of different grades of methylcellulose have become available which have a higher gel point than plain methylcellulose. These compounds are hydroxypropyl methylcelluloses made by the Dow Chemical Company and designated as Methocel 60 HG, Methocel 65 HG, Methocel 70 HG, and Methocel 90 HG. The number refers to the approximate gel point of a 2 per cent solution in degrees centigrade.

The purpose of this investigation was to determine the effect of additives on the gel point of various grades of Methocel[2]. The investigation concerned itself mainly with additives of pharmaceutical interest and of nonelectrolyte nature; since the effect of electrolytes is well known. Particularly, it was desirable to find suitable additives which would raise the gel point of methylcellulose solutions. Such compounds could be useful in preventing the gelling of products containing relatively high concentrations of methylcellulose and also in counteracting the gel point depressing effect of many compounds.

EXPERIMENTAL

Materials.—The following grades of Methocel were used in the investigation: Methocel, Methocel 65 HG, and Methocel 90 HG. All three grades were of the 4000-viscosity type. All other materials were U. S. P. grade; except magnesium chloride, which was reagent grade; and diethanolamine (Eastman), and sorbitol (Atlas).

Preparation of the Solutions.—Four per cent w/v stock solutions of the various Methocels were

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This study was in part conducted under a contract with the Armed Services Medical Procurement Agency.
Presented to the Scientific Section, A. PH. A., New York meeting, May 1957.

[1] Methocel (Dow Chemical Co.) was used in this study.

[2] The term "grade" as used in this paper refers to the degree and kind of substitution in the cellulose molecule.

prepared by adding the gum to distilled water previously heated to about 80°. The mixture was agitated for several minutes and then put into a refrigerator overnight; then it was again stirred for several minutes.

The various test samples were made from the stock solutions in the following manner: a sufficient amount of distilled water was added to the indicated amount of additive to make a final volume of 50 ml.; this was then added to 50 ml. of the stock solution to give a 100-ml. solution containing 2% Methocel.

Gel Point Determination.—A proper amount of the sample was placed in a 50-ml. centrifuge tube and any bubbles present were removed by centrifugation. The tube was then attached to a Brookfield Synchro-Lectric Viscometer, model LVF. A No. 3 spindle was used at 6 r. p. m. The centrifuge tube was immersed in a 2000-ml. beaker of water, which was slowly heated. An electric stirrer was used to keep the temperature equal throughout the bath. The temperature of the system was determined by placing a thermometer into another centrifuge tube which contained the same material and which was also placed in the beaker. Viscosity readings were taken at intervals of increasing temperature until the gel point was reached.

Gelling occurred in most cases quite abruptly, which made it easy to establish the gel point. However, some solutions, mainly those of Methocel 90 HG, failed to form a rigid gel, yet became cloudy and showed a marked viscosity increase. In order to obtain a satisfactory reference point for the solutions that did not gel abruptly, we have arbitrarily defined the gel point as the temperature at which the viscosity of the Methocel solution equals its viscosity at 35°. Two solutions made with plain Methocel gelled below 35°. Here the change to a rigid gel was abrupt, however, so that the gel point could be clearly established.

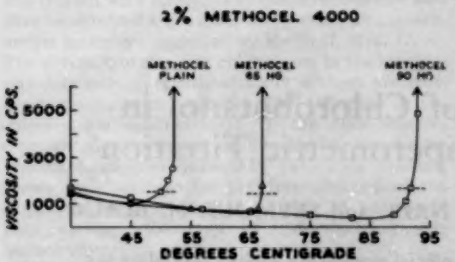

Fig. 1.—Viscosity-Temperature curves of 2% solutions of Methocel 4000, Methocel 4000 65 HG, and Methocel 4000 90 HG. Intercept of dotted line is the gel point.

RESULTS AND DISCUSSION

Figure 1 shows typical viscosity-temperature curves of 2% solutions of three grades of Methocel 4000: plain, 65 HG, and 90 HG. The viscosity decreases with increasing temperature until an abrupt viscosity increase indicates that gelling has occurred. The graph shows the higher gel points of the 65 HG and 90 HG grades as compared to that of plain Methocel.

The sharpness of the sol-gel transformation will be affected by the size-distribution range of the polymer (2) and by the degree of hysteresis (3). The first factor is probably somewhat different for each lot of gum. We have attempted to minimize hysteresis by heating the solutions very slowly by about 1° per minute. Since the manufacturers' specifications permit Methocel 4000 to have a viscosity range of 3000 to 5000 c. p. s., it is possible that the gel point will differ slightly from lot to lot.

Table I lists the gel points of 2% solutions of the Methocels tested in the presence of various additives. Gel points above 80° were not determined except in one instance: Methocel 90 HG.

TABLE I.—GEL POINT OF 2% METHOCEL 4000 SOLUTION

Additive	MC plain	MC 65 HG	MC 90 HG
None	50	67	92
5% Mag. chloride	43	56	59
20% Sorbitol	30	46	48
20% Sucrose	44	59	61
20% Glycerin	34	60	65–70
20% Ethanol	>75	>75	>75
20% P. E. G. 400	52	>80	>80
20% Prop. glycol	59	>80	>80

While the gel point lowering effect of electrolytes, such as magnesium chloride, has been mentioned earlier, the data also show that sorbitol, sucrose, and glycerin cause a decrease in the gel point. On the other hand, ethanol, propylene glycol, and polyethylene glycol 400 caused a significant rise of the gel point; except the last mentioned solvent showed little effect on the gel point of plain Methocel. Such an effect has been reported previously for ethanol (1, 4), Armac (Armour Company), an acetate of a high molecular weight amine, Hyamine 1622 (Rohm and Haas Company), alkali metal thiocyanates, and urea (4). None of the additives previously known to raise the gel point, with the exception of ethanol, is suitable for inclusion in pharmaceutical products in the concentrations required.

Since most additives lower the gel point, the possibility of using gel point increasing additives to overcome the effect of gel point lowering compounds was examined. Table II shows the effect of propylene glycol in overcoming the gel point depression due to magnesium chloride or sucrose. Since polyethylene glycol 400 also raises the gel point, it may be used likewise.

TABLE II.—GEL POINT OF 2% METHOCEL 4000 SOLUTION

Composition	MC plain	MC 65 HG	MC 90 HG
No additives	50	67	92
5% Mag. chloride	43	56	59
5% Mag. chloride and 20% Prop. glycol	45	66	>80
20% Sucrose	44	59	61
20% Sucrose and 20% Prop. glycol	48	69.5	>80

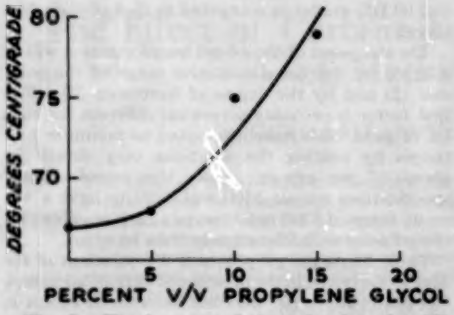

Fig. 2.—The increase of the gel point of a 2% solution of Methocel 4000 65 HG with increasing concentration of propylene glycol.

The effect of additives on the gel point is of course a function of their concentration. Figure 2 shows the increase of the gel point with increasing concentration of propylene glycol in a 2% solution of Methocel 4000 65 HG.

The reasons for the changes in gel point of Methocel solutions due to the presence of additives deserve further attention. The gel point depression by electrolytes is considered due to dehydration of the polymer (1) with a subsequent association of the dehydrated polymer chains with one another to form a gel. The gel point depression in the presence of sucrose, sorbitol, or glycerin can also be explained as being due to this dehydration effect, since these compounds are strongly hydrophilic.

However, the three compounds which raised the gel point, namely ethanol, propylene glycol, and polyethylene glycol 400 are also hydrophilic and should cause dehydration of methylcellulose. It is possible that the gel point increase is due to the solvent contribution of these compounds. As Table III shows, the effect of these additives on the gel point is proportional to their solvent action on Methocel. The term "solubility point" used in the table refers to the temperature in degrees C. at which one gram of Methocel 60 HG dissolves in 25 ml. of the pure solvent.

TABLE III.—2% METHOCEL 4000 SOLUTION

Additive	Gel Point MC Plain	Gel Point MC 65 HG	Solubility Point of MC 60 HG*
20% Prop. glycol	59	>90	140
20% P. E. G. 400	52	85	150
20% Diethanolamine	47	67	180
20% Glycerin	34	60	260
20% Sorbitol	30	46	Not soluble

* Reported by The Dow Chemical Company.

The additives which raised the gel point were relatively good solvents of Methocel, while the additives which depressed the gel point were relatively poor solvents, or did not dissolve the polymer at all. While this is not conclusive evidence, the data suggest the hypothesis that these compounds raise the gel point of Methocel solutions because they are relatively good solvents of the gum.

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The Determination of Chlorobutanol in Pharmaceuticals by Amperometric Titration*

By JOHN L. LACH, DAMODARAN NAIR, and SEYMOUR M. BLAUG

The determination of chlorobutanol by the official method is a lengthy procedure not readily applicable to pharmaceutical preparations. Chlorobutanol has been determined turbidimetrically, but sodium chloride and other halides interfere. A procedure for the direct determination of chlorobutanol in pharmaceutical preparations by the amperometric titration of chloride ion with silver nitrate using a rotating platinum electrode has been developed. This procedure is rapid and is not interfered with by sodium chloride, sodium bisulfite, and other halides.

CHLOROBUTANOL IS WIDELY USED in pharmaceuticals as a bacteriostatic agent. Its local anesthetic action can be considered as an asset to its use in parenteral solutions. A survey of the literature shows that most of the methods used for the quantitative determination of chlorobutanol are based on the liberation of chloride ion from chlorobutanol after treatment with alkali. The chloride ion is then determined vol-

* Received May 3, 1957, from the State University of Iowa, College of Pharmacy, Iowa City.
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umetrically, gravimetrically, or turbidimetrically.

Vastagh (1) and Ray and Basu (2) described methods whereby chlorobutanol was first distilled from the pharmaceutical and the distillate, after treatment with alkali, was titrated argentimetrically or iodometrically. Jensen and Jannke (3) described a procedure for the assay of chlorobutanol in the free state based on its decomposition with alkali and back-titrating the residue alkali. They also reported on an assay based on the hydrolysis of chlorobutanol, distillation with the aid of water vapor, and determination of the acetone in the distillate by an iodometric method. F. C. Sinton (4) weighed silver chloride obtained by the addition of alkali and excess silver nitrate and determined chlorobutanol gravimetrically. Taub and Luckey (5) estimated chlorobutanol turbidimetrically. A method for the determination of chlorobutanol by argentimetric titration is described in the U. S. P. XV (6).

The above described methods do not lend themselves readily to the quantitative determination of small quantities of chlorobutanol. Such a determination is necessary for a kinetic study of chlorobutanol. The purpose of this study is to develop a procedure such as can also be applied to the analysis of chlorobutanol in pharmaceutical preparations.

EXPERIMENTAL

In this method, as in others described in the literature, the sample containing the chlorobutanol was treated with alkali. The liberated chloride ions were determined argentimetrically using an amperometric procedure described by Kolthoff, *et al.* (7, 8). The presence of any free chloride ion in the samples was determined argentimetrically without pretreating the sample with alkali. The difference in the chloride ion concentration of the two samples represented the amount of chloride ion due to the breakdown of chlorobutanol by the alkali. Table I shows data obtained for the determination of the chlorobutanol content of a number of parenteral preparations. Recovery of chlorobutanol was quantitative.

Reagents and Apparatus.—Sargent Ampot amperometer; rotating platinum electrode; microburet, calibrated to deliver 0.01 ml. per drop; 0.08 *N* alcoholic silver nitrate solution; 5% sodium hydroxide solution; acetone; saturated ammonium nitrate solution; 1% gelatin solution; concentrated nitric acid; and chlorobutanol U. S. P., containing 4.84% moisture as determined with a Beckman aquameter.

Procedure.—Approximately 0.150 Gm. of chlorobutanol was accurately weighed in 50-ml. volumetric flasks and dissolved in approximately 15 ml. of alcohol. To each flask was added the ingredients corresponding to a formula in Table I and sufficient deionized water to make 50 ml. Each flask was analyzed in the following manner. A 2-ml. sample was pipetted into a 50-ml. beaker using a delivery pipet which was rinsed with acetone, the rinse being added to the beaker. To the beaker was added 20 ml. of acetone—to decrease silver chloride going into solution; 1 ml. of a saturated ammonium nitrate solution—to furnish sufficient electrolyte for the passage of current; 2 drops of a 1% gelatin solution—to coat the silver chloride particles and prevent possible reduction of silver ion at the electrode (7); and 1 drop of concentrated nitric acid—to prevent the formation of silver oxide. The mixture was titrated amperometrically with a rotating platinum electrode using 0.08 *N* alcoholic silver nitrate solution.

In order to obtain the total chloride ion concentration, that is, the free chloride ions in solution plus the chloride ions of the chlorobutanol, another 2-ml. aliquot of the sample solution was pipetted into a 50-ml. beaker. Two milliliters of a 5% solution of sodium hydroxide was added and the sample was allowed to react for approximately fifteen minutes. At the end of this time, the sample was treated and titrated as previously described except 8–10 drops of concentrated nitric acid was added to neutralize the excess sodium hydroxide. The difference in the ionic chloride content of the two samples corresponded to the amount of chloride ion due to the chlorobutanol present in the sample.

TABLE I.—ANALYSIS OF PARENTERAL PREPARATIONS FOR CHLOROBUTANOL CONTENT

	Formula	Gm./50 cc.	Recovery,[a] %
1.	Chlorobutanol	0.150	99.4
2.	Morphine Sulfate	0.150	
	Sodium Bisulfite	0.050	
	Chlorobutanol	0.150	99.4
3.	Procaine Hydrochloride	0.500	
	Sodium Chloride	0.318	
	Chlorobutanol	0.150	100.8
4.	Nicotinic Acid	0.500	
	Chlorobutanol	0.143	99.8
5.	Codeine Sulfate	1.500	
	Chlorobutanol	0.159	100.1
6.	Chlorprophenpyridamine Maleate	1.000	
	Chlorobutanol	0.148	100.3
7.	Sodium Iodide	5.000	
	Sodium Bisulfite	0.066	
	Chlorobutanol	0.152	100.1
8.	Methapyrilene Hydrochloride	1.000	
	Chlorobutanol	0.150	99.6
9.	Sodium Benzoate	3.125	
	Caffeine	3.125	
	Chlorobutanol	0.151	100.3

[a] Average of four determinations.

DISCUSSION

It is evident from the results obtained that this procedure is applicable to the determination of the chlorobutanol content of many pharmaceuticals. Organic halide compounds are also suited to this procedure, formula 6—Table I, unless the compound itself liberates halide ion on treatment with alkali. However, it would be possible in such cases to extract the chlorobutanol

and then determine it amperometrically after treatment with alkali. Inorganic halides such as sodium chloride and sodium iodide do not interfere in the determination of chlorobutanol. Results of a kinetic study of chlorobutanol solutions now underway in our laboratories will be presented in a future report.

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The Chromatographic Separation and Determination of Diphenylhydantoin and Phenobarbital*

By JOHN L. LACH, KANTILAL BHANSALI, and SEYMOUR M. BLAUG

A chromatographic method has been developed for separating diphenylhydantoin and phenobarbital on a partition column buffered to an alkaline pH. The two compounds are quantitatively determined spectrophotometrically after elution. The method has been applied to combinations of diphenylhydantoin and phenobarbital in capsules.

DIPHENYLHYDANTOIN SODIUM and phenobarbital are used extensively in the treatment of epilepsy. Although there are a number of methods (1–4) for the individual determination of diphenylhydantoin and phenobarbital, the analysis of a combination of these two compounds presents many difficulties. Spectrophotometric absorption procedures give only approximate determinations since diphenylhydantoin and phenobarbital absorb over a wide pH range in the ultraviolet region (5–8). The cobaltamine reaction (4) used for the quantitative determination of diphenylhydantoin is also positive for phenobarbital. A semiquantitative paper chromatographic method has been recently reported for the separation and determination of these two compounds (9). A complex extraction procedure for the determination of diphenylhydantoin in phenobarbital combinations has been reported (10).

The purpose of this study is to develop an analytical procedure for the separation and quantitative determination of diphenylhydantoin and phenobarbital.

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EXPERIMENTAL

The method developed in this study is based on the isolation of diphenylhydantoin and phenobarbital by partition chromatography and their determination in the effluent by spectrophotometric procedures.

Preliminary investigation of this separation procedure using Celite® 535[1] partition columns containing phosphate buffers, pH 3–7, as the internal phase indicated that the difference in the solubility behavior of diphenylhydantoin and phenobarbital in a chloroform external phase was not sufficient to effect a separation. Other solvent combinations showed no appreciable differentiation in the separation of these two compounds.

Partition columns buffered to an alkaline pH were investigated since the solubility of diphenylhydantoin and phenobarbital differs considerably in an alkaline medium. An internal phase buffered to a pH of 9.2 gave sharp separation using chloroform as the eluent for diphenylhydantoin and a 10:90 (v/v) *n*-butyl alcohol-chloroform mixture for phenobarbital, see Fig. 1. The concentration of diphenylhydantoin and phenobarbital in the effluents was determined at 266 mμ. This wavelength was determined from spectrograms of diphenylhydantoin and phenobarbital in their respective solvents.

Reagents and Apparatus.—Celite 535, reagent grade *n*-butyl alcohol and chloroform, 0.15 *M* borate buffer pH 9.2, 0.3 *M* hydrochloric acid, recrystallized phenobarbital U. S. P., recrystallized diphenylhydantoin, Pyrex glass chromatographic columns 45 cm. long and 2 cm. in diameter with glass plungers to pack the columns. Beckman DU spectrophotometer and 1-cm. silica cells.

Procedure.—The columns were prepared and packed in a manner previously described (11) using borate buffer pH 9.2 as the internal phase. Approximately 50 mg. of diphenylhydantoin and 30 mg. of phenobarbital was accurately weighed into a

[1] Celite 535 was supplied by the Johns-Manville Company.

50-ml. beaker. To this was added 3 ml. of distilled water and 3 Gm. of Celite 535. This was thoroughly mixed and a slurry was prepared by the addition of 5 ml. of chloroform. The slurry was transferred quantitatively to the column using 2 × 5-ml. portions of chloroform and was tightly packed on the surface of the column with a glass plunger which was rinsed

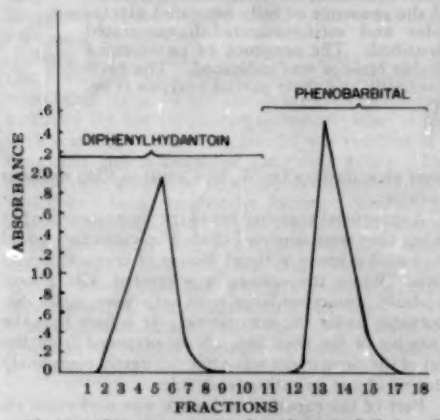

Fig. 1.—Chromatogram of a mixture of diphenylhydantoin and phenobarbital. Borate buffer, pH 9.2, was used as the internal phase; chloroform was used as the eluant for the first 100 ml.; 10:90 (v/v) *n*-butyl alcohol-chloroform mixture was used as the eluant for the remainder. Ten-milliliter fractions were collected.

with chloroform before removing from the column. The first 100 ml. of eluate was collected in a volumetric flask and its absorbance measured for diphenylhydantoin content. A second 100-ml. portion was collected using a 10:90 (v/v) *n*-butyl alcohol-chloroform mixture and its absorbance measured for phenobarbital content. The success of this method for various mixtures of diphenylhydantoin and phenobarbital is shown in Table I.

TABLE I.—DETERMINATION OF DIPHENYLHYDANTOIN AND PHENOBARBITAL IN KNOWN MIXTURES

Sample No.	Substance	Added to Each Column, mg.	Recovery, %
1	Diphenylhydantoin	48.93	99.80
	Phenobarbital	26.30	99.96
2	Diphenylhydantoin	30.55	99.90
	Phenobarbital	26.90	100.20
3	Diphenylhydantoin	50.76	99.88
	Phenobarbital	23.92	99.47
4	Diphenylhydantoin	64.89	98.99
	Phenobarbital	38.49	100.04

The content of 25 diphenylhydantoin sodium with phenobarbital capsules[1] was accurately weighed. Aliquots of this mixture containing between 50–90 mg. of diphenylhydantoin and 8–16 mg. of phenobarbital were accurately weighed into 50-ml. beakers. Three milliliters of 0.3 *N* hydrochloric acid was added to the beaker to obtain the diphenylhydantoin sodium as the free acid. The

[1] Supplied by Parke, Davis and Company.

TABLE II.—DETERMINATION OF DIPHENYLHYDANTOIN AND PHENOBARBITAL IN CAPSULES

Sample No.	Substance	Amounts (Labeled) Added in Each Column, mg.	Recovery,[a] %
1	Diphenylhydantoin	63.99	96.7
	Phenobarbital	11.12	104.9
2	Diphenylhydantoin	50.22	96.7
	Phenobarbital	8.73	108.0
3	Diphenylhydantoin	73.55	96.4
	Phenobarbital	12.79	108.4
4	Diphenylhydantoin	72.06	96.8
	Phenobarbital	12.53	109.6
5	Diphenylhydantoin	61.57	96.4
	Phenobarbital	10.64	106.5
6	Diphenylhydantoin	68.16	96.4
	Phenobarbital	11.85	104.2
7	Diphenylhydantoin	95.84	96.9
	Phenobarbital	16.64	104.5
8	Diphenylhydantoin	87.55	96.6
	Phenobarbital	15.23	107.1
	Av.—Diphenylhydantoin		96.5%;
	Phenobarbital		106.7%.

[a] Per cent recovery of diphenylhydantoin was based on the sodium salt.

same procedure, as outlined above, was then followed. Recovery data are shown in Table II. The percentage of recovery for diphenylhydantoin was based on the sodium salt.

DISCUSSION

A method for the separation and quantitative determination of diphenylhydantoin and phenobarbital is presented. The method is based on the separation of diphenylhydantoin and phenobarbital by partition chromatography using a borate buffer pH 9.2 as the internal phase. The variation in the percentage of recovery of phenobarbital, Table II, can be in part attributed to the low concentration of phenobarbital in the samples. Since the extinction coefficient for phenobarbital at 266 mμ is only 2.41 and since the determinations are made in the not too sensitive region of the spectrum, deviations of ±2 per cent are to be expected. However, if greater accuracy is desired for the phenobarbital determination it is recommended that an aliquot of pure phenobarbital be added to the sample.

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Analysis of Poison Ivy Fruit Fat*

By SADGURUNATH S. WARAWDEKAR† and PAUL J. JANNKE

The whole fat of poison ivy fruit was analyzed and its composition was determined. The fatty acids isolated included myristic, palmitic, stearic, linoleic, and oleic. The study of the glyceride composition disclosed the presence of fully saturated glycerides, disaturated-mono-unsaturated glycerides and monosaturated-diunsaturated glycerides. β-Sitosterol was isolated and identified. The presence of unsaturated hydrocarbons and pigments in the unsaponifiable residue was indicated. The pericarp fat and the seed oil were examined individually and their partial analysis is reported.

THE CHEMICAL INVESTIGATION of poison ivy dates back to 1858 when Khittel (1) described a volatile alkaloid which he erroneously reported to occur in the plant (2). Most investigators who have worked with the plant since that time were concerned with the isolation of its toxic principles. In so far as the nonpoisonous components of poison ivy are concerned, not much has been reported. Acree and Syme (3) described the isolation of gallic acid, fisetin, and rhamnose from the leaves and flowers. Stevens and Warren (4) described a waxy isolate from the mature fruits which had been deprived of pericarp. The yield was 33.4 per cent of material which had a specific gravity of 0.9577 at 25°, saponification value 238 and iodine number 30.1.

It was the purpose of this study to determine the chemical composition of the fatty extract from the mature fruits of the plant.

INTRODUCTION

The poison ivy fruits used in this investigation were gathered in the vicinity of Storrs, Conn., during late September of 1953 and 1954. Some of them were obtained from vines growing along stone walls while others were gathered from vines growing high in old apple trees. At the time of harvest, the fruits were mature as indicated by the fact that the yellowish epicarp had dried and begun to open, disclosing the ashen-white seeds within. Clusters of the fruits were thoroughly air-dried whereupon the stems were separated and the fruits were carefully rubbed over a coarse screen to dislodge the epicarp. During these operations the hands of the worker were protected by plastic gloves. About 5 lb. of clean fruits were available for the investigation. The plants from which they were obtained were identified by Dr. W. H. Camp[1] as *Rhus radicans radicans*.

A superficial examination of the fruits showed that when they were deprived of their epicarps they could be handled freely without danger of contact dermatitis. When the epicarp was treated with choral hydrate, numerous large resin cells were easily discernable under the microscope. It is here that the toxicity of the fruit lies. Soap prepared from the fat of the clean fruits was a fine detergent, completely devoid of any irritating tendencies.

Part of the experimental work was performed on the total lipoidal matter which was extractable from the entire fruits which had been freed of the epicarp. Actually, the lipoidal matter was of two sources, namely pericarpal fat and seed oil. The former was a white, waxy substance while the latter was a golden yellow oil. Since these two components were so different in nature, they were isolated and examined individually.

EXPERIMENTAL

Preliminary examination of the fruit showed a low moisture content and a high fat content. Air-dried samples lost 3.68% of their weight when dried for one hour at 100°. Complete extraction with low boiling petroleum ether yielded 29.7% fat.

Dragendorff Extraction.—Duplicate 20-Gm. samples of clean, air-dried fruits were exhaustively extracted with a series of solvents, in the usual manner. The results are given in Table I.

TABLE I.—EXTRACTION WITH SELECTIVE SOLVENTS

Solvents	Extractive, % I	II
Petroleum ether	29.75	29.66
Absolute ether	1.53	1.34
Chloroform	0.41	0.26
Absolute ethyl alcohol	0.73	0.71
70% ethyl alcohol	3.80	3.60
Cold distilled water	1.66	1.63
Boiling distilled water	1.07	0.91
Cold 1% solution of hydrochloric acid	1.68	1.81
5% solution of sodium hydroxide	27.90	27.54

Each extract was evaporated to dryness on a steam bath. All of the residues, except for the alkali extractive, were examined for the presence of alkaloids, but no positive tests resulted. The residues obtained by petroleum ether, absolute ether, and

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chloroform extraction were each washed with NaOH solution and the aqueous phase was treated with benzenediazonium chloride at 0°. Failure of the coupling reaction indicated the absence of phenols. The residues of the two alcoholic extracts yielded orange precipitates under the same conditions, indicating the presence of phenolic matter, probably tannin. These extracts also contained polysaccharides which reduced Fehling's solution after inversion.

Extraction of Fat.—A large quantity (1927 Gm.) of clean, dried fruit was extracted with petroleum ether (b. p. 30–60°) in an all-glass continuous extractor for fourteen hours to remove most of the pericarpal fat. The marc (seeds) was crushed in a drug mill and re-extracted for thirty hours. The solvent was recovered from the combined extracts which had been clarified by filtration through an acid-washed sand bed. Final traces of the solvent were removed by bubbling CO_2 through the warm residue. The yield was 586 Gm. (29.71%) of a grayish-white solid fat which possessed a mild, bland odor. It was refrigerated under CO_2.

Examination of the Fat.—The fat of poison ivy fruit did not contain nitrogen or sulfur, indicating the absence of sulfatides and amino lipids. Its refractive index was 1.4560 at 40°. The specific gravity, determined by the pycnometer method was 0.8567 at 99°/25°.

The chemical constants of the fats were determined according to established procedures (5) (see Table II).

Saponification of the Fat.—One hundred Gm. of fat was refluxed with 30 Gm. of KOH in 500 ml. of ethanol to effect saponification. After adding water, most of the alcohol was recovered and the unsaponifiable matter was separated by extraction with ether.

The residual soap solution, freed of ether, was acidified with an excess of dilute H_2SO_4 and the resulting mixture was warmed until the liberated acids melted and formed a clear supernatant layer. This was separated and the aqueous phase was extracted with ether. The acids previously isolated were dissolved in the combined ethereal washings, and the solution was dried over anhydrous Na_2SO_4. Recovery of the solvent yielded the total fatty acids, weighing 88.0 Gm.

Separation of Saturated and Unsaturated Fatty Acids.—The Bertram method (6) for the quantitative determination of saturated fatty acids was attempted but difficulty was experienced in dissolving the total fatty acids in petroleum ether. The failure of the method however, indicated that there is a predominance of acids having fewer than 16 carbon atoms, and this was borne out subsequently (see Table IV).

The separation of the fatty acids was accomplished by application of the classical lead salt–ether method (5). A 60-Gm. portion of total fatty acids yielded 44.18 Gm. of saturated acids and 15.75 Gm. of unsaturated acids, corresponding to 73.63% and 26.37% respectively. The physical and chemical constant of the two acid fractions are shown in Table III.

Saturated Fatty Acids.—The saturated fatty acids (30.0 Gm.) were converted to their methyl

TABLE II.—CHEMICAL CONSTANTS OF POISON IVY FRUIT FAT

	I	II
Acid value (mg. KOH/1 Gm. of fat)	7.19	7.43
Saponification value	205.90	206.20
Iodine number (Hanus)	35.01	35.30
Iodine number (Wijs)	35.68	35.89
Thiocyanogen value	23.11	23.06
Hehner value	91.20	91.17
Reichert-Meissl number	0.87	0.78
Polenske value	1.39	1.49
Acetyl value	2.99	3.13
Unsaponifiable matter	0.89	0.91

TABLE III.—CHEMICAL AND PHYSICAL CONSTANTS OF THE TOTAL, SATURATED AND UNSATURATED FATTY ACIDS

	Molecular Weight	Iodine Value	Thiocyanogen Value	Titer, °C.	Melting Point, °C.	Refractive Index, °C.
Total fatty acids	245.84	38.47 38.17	24.44 24.23	46.5	40	1.4412 at 60
Saturated fatty acids	243.70	1.02 1.01	...	50.4	43.5	1.4355 at 60
Unsaturated fatty acids	276.41	132.60 132.00	83.05 82.77	..	..	1.4640 at 25

TABLE IV.—FRACTIONAL DISTILLATION OF METHYL ESTERS OF SOLID ACIDS[a]

Fraction	Weight, Gm.	Boiling Point at 5 mm. Pressure, °C.	Refractive Index at 45°	Saponification Equivalent	Iodine Value	Calculated Composition of Ester Fractions			
						Saturated			Oleic and Linoleic
						C_{14}	C_{16}	C_{18}	
1	14.5	Up to 143	1.4274	241.8	..	14.5	..	..	..
2	12.5	143–175	1.4318	270.1	0.65	..	12.41	..	0.09
3	2.5	175–189	1.4420	293.7	9.7	..	1.0	1.77	0.03
and Residue	0.3								

[a] Weights—14.5, 13.41, 1.77, 0.12; per cent esters—48.66, 45.00, 5.96, 0.38; per cent acids—48.66, 45.00, 5.96, 0.38.

esters (30.08 Gm.), and the mixed derivatives were fractionated at 5 mm. pressure in a Todd precise fractionation assembly (see Table IV).

Fraction one was hydrolyzed with alcoholic KOH and the acid thus liberated was isolated and purified. Its molecular weight, determined through the neutralization equivalent (229.9), corresponded to that of myristic acid (mol. wt. 228.36). To further confirm its identity, the acid was converted to the hydroxamate which melted at 97.5–98.5°. Myristohydroxamic acid melts at 98–98.5°.

Fraction two, on hydrolysis with alcoholic KOH, yielded an acid whose neutralization equivalent was 254.7, which compares favorably with the molecular weight of palmitic acid (256.42). The hydroxamate of the acid melted at 102–103°. Palmitohydroxamic acid melts at 102.5°.

Fraction three and the residue were combined and hydrolyzed as above. The acid recovered had the molecular weight 280.2, which compares with that of stearic acid (M. W. 284.47). The hydroxamate, melting at 105–106°, was concluded to be stearohydroxamic acid, m. p. 106–107°.

Unsaturated Fatty Acids.—The separation of the unsaturated fatty acids (1.1 Gm.) was accomplished by the standard bromination procedure. The absolute ether solution of the mixed bromides yielded no crystalline matter, indicating the absence of linolenic acid. This is confirmed by the thiocyanogen number (Table III).

The petroleum ether solution yielded 0.66 Gm. of tetrabromolinoleic acid, m. p. 115°, corresponding to 28.0% of the unsaturated acid fraction.

Dibromo-oleic acid recovered from the petroleum ether filtrate was debrominated with zinc dust in the usual manner, yielding 0.85 Gm. of oleic acid, corresponding to 72.0% of the unsaturated acid fraction. Its iodine value was 85.48 and its molecular weight was 280.

TABLE V.—COMPOSITION OF THE MIXED FATTY ACIDS

Acids	Weight, %	Molar, %
Myristic	36.0	32.33
Palmitic	33.1	33.37
Stearic	4.5	5.03
Oleic	20.1	22.32
Linoleic	6.3	6.95

Component Glycerides of the Whole Fat.—The saturated glycerides were isolated from the whole fat by the method of Hilditch and Lea (7). Following permanganate oxidation in dry acetone, 17.64 Gm. of neutral fat yielded 9.49 Gm. of saturated glyceride, corresponding to 48.13% of the neutral fat. Its chemical constants were: saponification value 217.0, iodine value 0.0, acid value 0.4.

Based upon the selectivity of $KHCO_3$ and K_2CO_3 solutions, Hilditch (8) described the resolution of the oxidation products produced in the determination of saturated glycerides. Mono- and diazelaoglycerides were thus isolated from the reaction mixture, however, no triazelaoglyceride was found. Kartha and Menon (9) used the saponification value of these cleavage products to calculate the mono- and diunsaturated glyceride content of a fat. The yield of mono- and diazelaoglyceride was 4.5333 Gm.; saponification value 316.2. The projection of these data in the manner described by Kartha and Menon gave rise to the percentage composition of the glycerides of poison ivy fat (see Table VI).

TABLE VI.—COMPONENT GLYCERIDES OF THE POISON IVY FRUIT FAT

Glycerides	Weight, %
Trisaturated	48.13
Disaturated monounsaturated	22.01
Monosaturated diunsaturated	29.86

Unsaponifiable Matter.—The ethereal solution of the unsaponifiable matter was alternately washed with dilute NaOH solution and water. After drying over anhydrous Na_2SO_4, the solvent was recovered under reduced pressure, leaving a residue which weighed 0.776 Gm., corresponding to 0.776% of the whole fat.

A portion of this material (225.1 mg.) dissolved in a minimum of petroleum ether was placed on a column (2 x 30 cm.) of adsorption alumina (Fisher Scientific Co., 80–200 mesh). The column was successively eluted with 150 ml. each of petroleum ether, carbon tetrachloride, benzene, and chloroform containing 10% ethanol. The extracts were freed of their solvents, dried in a vacuum, and weighed.

The petroleum ether extract (30.4 mg.) was a colorless liquid which congealed when cooled slightly and which was soluble in concentrated H_2SO_4. It decolorized bromine water and had an iodine value of 20.44; $n_D^{25} = 1.4700$. Presumably it was a hydrocarbon (10, 11) but it was not identified.

The carbon tetrachloride eluate yielded 11.2 mg. of brown, waxy material. Although waxes have been reported in this fraction (10), the amount was too small for identification.

The benzene eluate contained pigments as could be judged from its deep yellow color. The residue (13.3 mg.) was rechromatographed in the hope of isolating carotenes (5). Spectrophotometric analysis failed to give a rational absorption pattern, whereas a chloroform solution of standard β-carotene gave an absorption maximum at 462 mμ.

The chloroform-ethanol eluate yielded 161.6 mg. of residue which responded strongly to the Liebermann-Burchard test. An alcoholic solution of it was purified by filtration through charcoal. The clear filtrate deposited lustrous white platelets melting at 136.5–137°. Purification by chromatography and fractional recrystallization gave only one compound melting at 137°. This melting point corresponds with that of β-sitosterol. The identity of the compound was confirmed by the preparation of its acetate (m. p. 125°) and its benzoate (m. p. 147°), both of which compare favorably with the corresponding derivatives of β-sitosterol.

Pericarp and Seed Fat.—It was previously stated that the whole fat actually consisted of two portions, namely that from the pericarp and that from the seeds. The former is a soft, grayish-white mass which completely envelops the dark colored, stony seed; whereas the latter is an oil.

Working in batches of 200 Gm., a large quantity of clean, whole poison ivy fruits (devoid of epicarp)

was alternately washed with petroleum ether and rubbed against a No. 10 sieve to remove all of the pericarpal fat. The solid fragments together with the residue from recovery of the solvent corresponded to 24.2% of the weight of the whole fruits.

The seeds were dried and then lightly crushed in a drug mill and extracted for ten hours with petroleum ether. The marc was reground and re-extracted. Recovery of the solvent from the combined extracts yielded a clear, golden-yellow oil. The oil content of the seeds was 9.0%, corresponding to 5.5% of the whole fruit.

Both the pericarpal fat and the seed oil were examined in the usual manner, and the results are given in Tables VII and VIII.

TABLE VII.—FAT FROM THE PERICARP PORTION OF THE POISON IVY FRUIT

Physical and Chemical Constants	
Refractive index at 60°	1.4470
Specific gravity, 99°/25°	0.8512
Acid value (mg. KOH/1 Gm. of fat)	2.73
Saponification value	214.10
Iodine value	14.76
Hehner value	95.23
Unsaponifiable matter	0.88

The total fatty acids of the epicarp fat consisted of 88.0% saturated acids and 12.0% unsaturated acids.

TABLE VIII.—OIL FROM THE SEEDS OF THE POISON IVY FRUIT

Physical and Chemical Constants	
Refractive index at 25°	1.4690
Specific gravity, 25°/25°	0.8762
Acid value (mg. KOH/1 Gm. of the oil)	2.2
Saponification value	177.8
Iodine value	125.6
Hehner value	90.53
Unsaponifiable matter	2.04

The total fatty acids of the seed oil consisted of 6.4% saturated acids and 93.5% unsaturated acids.

SUMMARY

Since an extensive investigation on poison ivy plant sap has already been made, the chemical analysis of the lipids of the fruits of poison ivy (*Rhus radicans radicans*) was undertaken to obtain a clearer picture of this notorious plant.

The poison ivy fruit fat obtained by solvent extraction was found to be 29.75 per cent of the whole fruit and was devoid of sulfatides and amino lipids. It is a grayish-white solid soft mass possessing a mild, bland odor and it does not give any specific color test.

The fatty acids were separated by lead salt–ether method and the individual saturated acids were obtained by fractional distillation of the methyl esters under reduced pressure. The individual unsaturated acids were separated by bromination. The mixed fatty acids were found to contain 36.0 per cent myristic acid, 33.1 per cent palmitic acid, 4.5 per cent stearic acid, 20.1 per cent oleic, and 6.3 per cent linoleic acid.

The glyceride composition of the fat was determined by the acetone–potassium permanganate oxidation method. The fully saturated glycerides were found to be 48.13 per cent, disaturated-monounsaturated 22.01 per cent, and monosaturated-diunsaturated 29.86 per cent.

The unsaponifiable matter which comprised about 0.9 per cent of the fat consisted of hydrocarbon, waxy material, pigments, and a sterol. The sterol was identified as β-sitosterol. Pigment and waxy material were not investigated for lack of sufficient material. Hydrocarbon appeared to be unsaturated, having iodine value 20.44 and refractive index 1.470 at 40°.

Pericarp fat was separated manually and was found to represent 24.2 per cent of the fruit. The mixed fatty acids of this fat contained 88 per cent solid acids and 12 per cent unsaturated fatty acids. Solid acids were individually separated by fractional distillation and were found to consist of myristic and palmitic acids as major components.

The seed oil represented 5.5 per cent of the weight of the fruit. The unsaturated acids in this oil amounted to 93.53 per cent of the total fatty acids.

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A Study of Moisture Vapor Transmission through Closures*

By SEYMOUR M. BLAUG, EUGENE HICKMAN, and JOHN L. LACH

Moisture vapor transmission through closures is an important consideration when packaging pharmaceutical products. It is particularly important when packaging hygroscopic materials or materials that decompose in the presence of trace amounts of moisture. The cap as well as the cap liner must be considered in choosing a proper closure. The barrier efficiency of sixteen liners in metal caps, phenolic, melamine, and mineral filled plastic caps was studied. Of the liners investigated, polyethylene, aluminum foil, plastic-wax saturated pulpboard, and red rubber liners were found to be the most effective moisture vapor barriers, particularly when used in metal closures.

THE SELECTION OF THE BEST possible closure for a specific product is of great importance in the drug and chemical industry. Some of the factors to be considered are the type of product to be packaged, its hygroscopicity, and product reaction with the cap and/or the cap liner. Although the liner may be effective and stable, the material of the cap may react with the product being packaged. This is particularly true of closures for liquid preparations.

A crucial problem for the packaging department is the selection of the best possible closure for dry, solid products that decompose in the presence of traces of moisture or are hygroscopic. Ascorbic acid tablets, for example, develop brown spots in a humid atmosphere due to the increased rate of oxidation of the ascorbic acid. The color and stability of many tablet coatings is affected adversely by the presence of moisture.

Metal caps are generally preferred to plastic caps as closures for dry products. High humidity can cause the expansion of plastic caps, particularly those made from a phenolic resin. The expansion of the cap produces "backoff" or a slow unscrewing of the cap (1). In a communication from Eli Lilly and Company it was stated that the company's standard closure for dry products is enameled tin plate, while the standard liner is a waxed paper applied to a pulp backing. Where extremely hygroscopic dry products are involved, the preferred liner is waxed aluminum foil over pulp.

It was the purpose of this study to compare the barrier efficiency of various liners in metal and plastic screw caps against moisture vapor transmission. Sixteen liners were studied in metal caps, phenolic, melamine, and mineral filled plastic caps.

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This research project was conducted under contract from the Armed Services Medical Procurement Agency, Brooklyn, N. Y.

EXPERIMENTAL

Materials and Apparatus.—Anhydrous calcium chloride, 11-dram emerald green glass vials, constant temperature humidity cabinet, semi-micro Gram-atic balance, Owens-Illinois torque tester, 28-mm. metal and plastic screw caps (Tables I and II).

Procedure.—Ten grams of anhydrous calcium chloride was placed into 11-dram vials. The screw caps under investigation were placed on each vial using the Owens-Illinois torque tester. Each cap was applied using 10-inch pounds torque. This gives removal torque values varying between 6 and 7-inch pounds.

The sealed vials were accurately weighed and placed upright in a constant temperature humidity cabinet adjusted to 38° ± 1° and a relative humidity of 90% ± 5%. The vials were removed from the humidity chamber at weekly intervals and allowed to equilibrate at room temperature for fifteen minutes before weighing. Immediately after weighing they were returned to the humidity chamber. Three vials were used for each test. Empty vials with caps were used as controls. It was found that the controls gained 9 mg. ± 1 mg. after one

TABLE I.—METAL CLOSURES* STUDIED

Number	Liner
1	0.003-in. polyethylene extruded on paper
2	0.0035-in. aluminum foil laminated to Kraft paper—foil side coated with a pigmented vinyl coating
3	Red rubber
4	Two sides coated paper bonded to wax saturated paper board
5	0.002-in. aluminum foil on paper board
6	Pulp and oil waxed
7	Pulp and vinyl
8	Plastic-wax saturated white pulpboard with coating 0.008–0.012 in. of a special plastic wax on one surface
9	Pulp and Saran film waxed
10	0.040-in. solid polyethylene
11	Pulpboard
12	0.035-in. pulp and Kraft paper backed with 0.001 in. Mylar polyester film

* Closures 1–9 were supplied by Crown Cork and Seal Company. Closures 10–12 were supplied by Armstrong Cork Company.

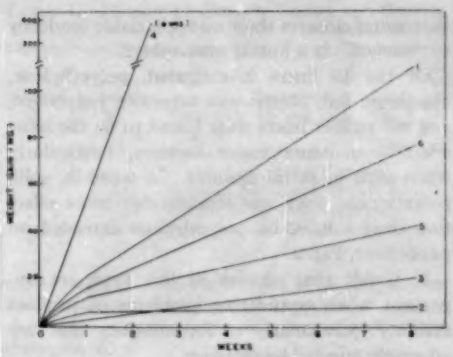

Fig. 1.—Moisture vapor transmission plots-metal closures.

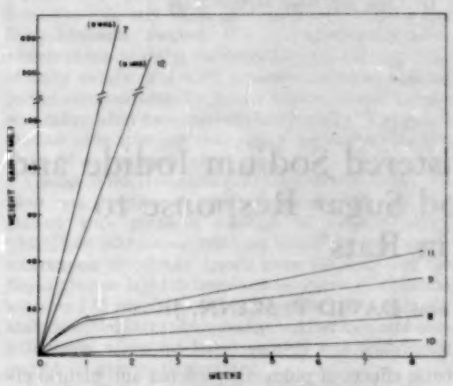

Fig. 2.—Moisture vapor transmission plots-metal closures.

TABLE II.—PLASTIC CLOSURES* STUDIED

Number	Type Plastic	Liner
13	Melamine	0.040-in. solid polyethylene
14	Phenolic	0.040-in. solid polyethylene
15	Mineral filled	0.040-in. solid polyethylene
16	Melamine	Pulpboard
17	Phenolic	Pulpboard
18	Mineral filled	Pulpboard
19	Phenolic	Polyethylene cone
20	Melamine	Mylar film laminate on pulpboard
21	Phenolic	Mylar film laminate on pulpboard
22	Mineral filled	Mylar film laminate on pulpboard
23	Phenolic	0.040-in. solid polyethylene
24	Phenolic	Aluminum foil
25	Phenolic	Pulp and vinylite waxed
26	Phenolic	Pulp and vinylite lubricant film

* Closures 13–18 and 20–22 were supplied by Armstrong Cork Co. Closure 19 was supplied by the Poly-Seal Corporation. Closures 23–26 were supplied by Owens-Illinois.

week and remained at this value for the eight-week period of the study. The weight gain in mg. each week is the average gain of the three vials used for each test. Results are shown in Figs. 1–4.

On completion of the study, all caps were removed with the torque tester. Removal torque for metal caps was 6–7 inch-pounds. For plastic caps, the removal torque varied depending on the type of plastic used. Phenolic resin plastic caps gave the lowest removal torques of 4–5 inch-pounds. Melamine and mineral filled plastic caps gave removal torques of 5–7 inch-pounds. The Poly-seal cap gave a removal torque of 6–7 inch-pounds.

DISCUSSION

Results of this study indicate that in general metal closures are more satisfactory than plastic closures as barriers to moisture vapor. The liner greatly affects moisture vapor transmission as does the type of plastic used in the closure. A 0.040-in. solid polyethylene liner in a metal closure is a more satisfactory barrier to moisture vapor than a 0.040-in. solid polyethylene liner in any of the three types of plastic closures used in this study, Figs. 2 and 3. Of the three types

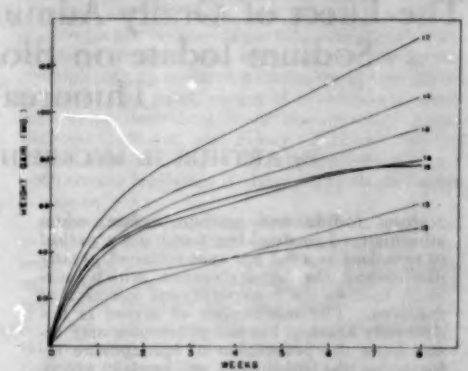

Fig. 3.—Moisture vapor transmission plots-plastic closures.

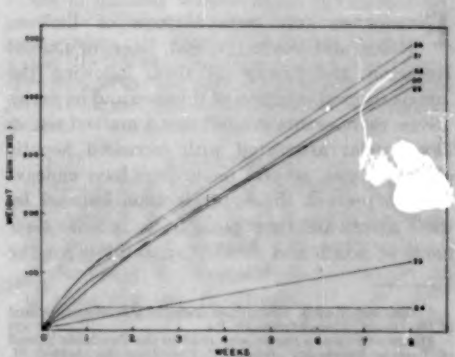

Fig. 4.—Moisture vapor transmission plots-plastic closures.

of plastic closures studied, melamine and mineral filled plastic closures are more effective barriers to moisture vapor than phenolic resin closures. Removal torque values indicate that the difference in barrier efficiency of the three types of plastic closures is probably due to the "backoff," or slow unscrewing of phenolic closures. This is caused by the expansion of phenolic resin closures in a humid atmosphere. The use of melamine or mineral filled plastic closures decreases this "backoff" tendency. One type of phenolic resin closure studied, the Poly-Seal closure, did not backoff to any appreciable extent. This closure consists of a plastic cap with a polyethylene cone liner which molds itself around the sealing areas of the container. As the closure is applied, the polyethylene liner takes the shape of the container finish. Removal torque values indicate that metal closures show no appreciable tendency to "backoff" in a humid atmosphere.

Of the 16 liners investigated, polyethylene, aluminum foil, plastic-wax saturated pulpboard, and red rubber liners were found to be the most effective moisture vapor barriers, particularly when used in metal closures. A 0.040-in. solid polyethylene liner was considerably more effective than a 0.003-in. polyethylene extruded on paper liner, Fig. 2.

It is felt that studies of this type are important when considering problems of product stability, particularly of formulations that are adversely affected by moisture.

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The Effect of Orally Administered Sodium Iodide and Sodium Iodate on Blood Sugar Response to Thiourea in Rats*

By ARTHUR H. MCCREESH and DAVID E. MANN, Jr.

Sodium iodide and sodium iodate, when administered in drinking water for a period of two days as a 0.2 per cent solution, greatly diminished the hyperglycemia which normally follows the intraperitoneal injection of thiourea. The mechanism of action is not definitely known, but the protection may result from the prevention of epinephrine release, or the inhibition of hepatic glycogenolysis.

FOLLOWING THE OBSERVATIONS of DuBois, Holm, and Doyle (1), and, later, of Dubois Hermann, and Erway (2) that, following the intraperitoneal injection of thiourea and its derivatives, there occurs in adult rats a marked rise in blood sugar associated with increased hepatic glycogenolysis, several researchers have endeavored to prevent the hyperglycemia induced by these agents and thus, possibly, to uncover their mode of action and develop an antidote for the toxic effects of pulmonary edema and pleural effusion, as seen in adult rats. The injection of iodide every four days has protected rats (3). Byerrum (4) and Mann (5) have shown that the oral administration of iodine and iodides, either as Lugol's solution, potassium iodide or potassium iodate, will similarly afford protection against the hyperglycemic response, and will enable the animal to withstand normally lethal doses.

This experiment is intended to determine the relative protective activity of sodium iodide and sodium iodate against the hyperglycemia following the intraperitoneal administration of ten milligrams per kilogram of body weight of thiourea to nonfasted, male, albino rats.

EXPERIMENTAL

Male rats of the Wistar strain were used exclusively in this experiment. The animals were placed in individual cages and were permitted to remain in these cages for periods of five to eight days before being used. All were fed Purina Lab Chow, and were given tap water *ad libitum* through glass tubes attached to drinking bottles until placed on a particular liquid diet. The same drinking bottles

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Presented to the Scientific Section, A. PH. A., New York City meeting, May 1957.

TABLE I.—EFFECT OF SODIUM IODIDE AND SODIUM IODATE ON BLOOD SUGAR RESPONSE TO THIOUREA IN NONFASTED, MALE, ALBINO RATS

Group	No. of Animals	Treatment	Av. Blood Sugar before Thiourea, mg./100 ml.	Av. Blood Sugar after Thiourea, mg./100 ml.	Av. Diff.	Av. Change, %
1	27	Thiourea 10 mg./Kg. (controls)	113.63	183.48	69.85 (±33.39)	63.67
2	12	Sodium Iodide *ad libitum* for two days, then thiourea	101.72	117.93	16.21 (±13.62)	19.70
3	12	Sodium Iodate *ad libitum* for two days, then thiourea	112.20	132.63	20.53 (±12.54)	18.90

were employed to deliver sodium iodide and sodium iodate solutions throughout the experiment. At the start of the experiment the animals were divided into three groups.

The rats in Group One, all nonfasting, male, albino rats, ranging in weight between 182 and 270 grams, were given tap water *ad libitum* as drinking water for two days, and served as controls in the experiment. After two days, the animals were bled from the tail, and blood glucose determined by the Folin-Malmros method (6). Immediately after withdrawing blood, a thiourea solution (10 mg./Kg. of body weight of a 0.5% aqueous solution) was injected intraperitoneally, and a second blood sample was taken after two and one-half hours. The blood glucose concentration was again determined by the Folin-Malmros method.

Group Two, consisting of nonfasted, male, albino rats, ranging in weight between 138 and 182 grams, was given a solution of sodium iodide (0.2%) *ad libitum* as drinking water for two days, whereupon blood was drawn from the tail, and the blood glucose level determined as above. Thiourea solution (10 mg./Kg. of body weight) was immediately injected intraperitoneally. After two and one-half hours a second blood sample was withdrawn and blood glucose determined.

Group Three consisted of nonfasted, male, albino rats, ranging in weight between 125 and 252 grams. These animals were treated identically as Groups One and Two, except that they received sodium iodate solution (0.2%) as drinking water *ad libitum* for two days. Blood samples were drawn from the tail both immediately before and two and one-half hours following the intraperitoneal injection of thiourea (10 mg./Kg. of body weight). Blood glucose was determined by the Folin-Malmros method as with all groups.

RESULTS

The rats in Group One, receiving no iodide or iodate therapy prior to the intraperitoneal injection of thiourea, showed an average blood sugar rise of 69.85 (±33.39) milligrams per 100 milliliters of blood two and one-half hours following the injection.

The rats in Group Two, receiving a two-day, *ad libitum* administration of sodium iodide prior to an intraperitoneal injection of thiourea, showed an average blood sugar rise of 16.21 (±13.62) milligrams per 100 milliliters of blood two and one-half hours after the injection.

The rats in Group Three, receiving a two-day *ad libitum* administration of sodium iodate prior to an intraperitoneal injection of thiourea, showed an average blood sugar rise of 20.53 (±12.54) milligrams per 100 milliliters of blood two and one-half hours after the injection.

DISCUSSION

The rodenticidal property of thiourea and its derivatives, proposed by Richter (7), and tested on a controlled scale by MacKenzie and MacKenzie (8), and others in the early and middle forties, is a result of severe pulmonary edema and pleural effusion. Drinker (9) found an eighty-fold increase in lymph flow from the heart and lungs following an injection of alphanaphthylthiourea (ANTU) into rats. Although toxic doses vary greatly among strains of the same animal, and wild rats are much more resistant than laboratory animals, there can be no doubt of the toxicity of these agents.

It was DuBois, Holm, and Doyle (1) who first observed a rise in blood sugar following the administration of thiourea and its congeners, the hyperglycemia being associated with increased liver glycogenolysis; the onset, extent of activity, and duration being dependent upon the quantity given. DuBois, Hermann, and Erway (2) noted that the results were not altered by hypophysectomy, nor was there an inhibition of glycolysis or the oxidation of glucose.

Although the mechanism of action is uncertain and as yet no specific antidote has been found for thiourea, it has been observed that drugs which prevent or diminish the hyperglycemic response will also prevent or diminish the toxic effects. Griesbach, Kennedy, and Purves (3) have protected animals by the repeated injection of potassium iodide; and Byerrum (4) enabled rats to withstand many times the lethal dose by feeding the animals a diet rich in iodine, and by administering potassium iodide and iodine in drinking water. Mann (5) showed the protective action of potassium iodide and potassium iodate when administered *ad libitum* in drinking water. In this experiment the protective properties of sodium iodide and sodium iodate against the hyperglycemic response to thiourea have been demonstrated.

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A Study of the Effect of Lubricant and Fines on a Tablet Granulation*

By F. C. HAMMERNESS† and HERMAN O. THOMPSON‡

A series of six formulations was made in which the amount of lubricant added was a variable ranging from 0 to 5 per cent, and in which the amount of fines added was a variable ranging from 0 to 70 per cent. The rate of flow was measured, using a standard procedure which was modified for this experiment. A measurable effect was induced by the lubricant and the fines. The study was extended to determine whether the effects of lubricant and of fines could be demonstrated in the actual compression of the tablet. The results obtained indicate that some measurable effects can be noted. The physical properties evaluated were: weight variation, hardness, thickness, disintegration, and durability. The method of determining durability of tablets was devised for this study, while the other methods used were standard procedures.

A REVIEW OF THE LITERATURE (1–59) reveals that most of the investigation and research on tablet manufacture, with the exception of studies on tablet disintegration and tablet coating, have been conducted since 1945. This publication does not attempt to review the literature beyond providing a selected bibliography on tablet manufacture, inasmuch as references on tablet coating and disintegration are excluded beyond the citation of the two references (15, 16) which adequately cover these phases of studies.

The effects that fines and lubricant have upon a granulation are known empirically by many workers who have had experience in tablet manufacture, but there is no significant quantitative data to substantiate many of the statements made about them. Textbooks and bulletins are similarily deficient as to specific information which could be greatly beneficial to the beginner. This work was conducted to contribute quantitative findings as to these effects.

PRELIMINARY INVESTIGATION

The granulation used throughout this study consisted of lactose employing a 2% solution of liquid glucose U. S. P. as the binding and wetting agent. A 16-mesh sieve was used for screening, so that all granules that passed through a 16-sieve but were retained by a 40-sieve were considered a #16 granulation. The portion which passed through the 40-sieve was considered fines. These fines were analyzed with the following results:

40 to 60 mesh	28%
60 to 80 mesh	22%
80 to 100 mesh	15%
Less than 100	35%

The above percentages were used in making "control fines" for the study. The #16 granulation contained no fines according to the above definition. Calculation of per cent fines added in each formulation was based upon the final total weight; that is, 10% fines was interpreted as 10 grams of fines and 90 grams of granulation.

Talc was used as a lubricant because of its general acceptance over the years and because of its economy and relative freedom from incompatibilities and adverse reactions. One gram of talc was used for each 100 grams of granulation, or 1% in this instance.

The density of the granulation determined in the conventional manner was found to be 1.505 as compared to 1.418 for lactose alone.

EXPERIMENTAL

To measure the effect of fines and lubricant upon tablet granulations, determinations were made both as to the rate of flow of the granulations and physical characteristics of the tablets compressed; furthermore a correlation was sought between the rate of flow and characteristics of the compressed tablets. A statistical analysis was performed on the data in an attempt to determine whether the results obtained could be expressed more quantitatively.

Flow Rate.—The rate of flow was measured by using a standard funnel and a measuring cup as described in A. S. T. M. Standard D-392-38(60). The stainless steel funnel measured $3^{31}/_{32}$ inches in diameter, $4^{1}/_{2}$ inches vertically from top to bottom, a $^{3}/_{8}$ inch inside diameter at the bottom and the side had a slope of 20 degrees from the vertical. The measuring cup was a glass cylinder with a capacity of 100 ml., calibrated to an accuracy of 0.5 ml. It measured 1.572 inches for inside diameter, and 3.144 inches for inside height.

The small end of the funnel was closed with the finger and a sample was poured into the funnel. The bottom of the funnel was quickly opened and the material was allowed to flow freely into the cup. After all of the material had passed through, the excess amount was leveled off with a spatula. The material in the cup was weighed to the nearest 0.1 gram. This amount of the granulation was placed in the funnel, and at the same instant that the bottom was opened a stop watch was started. The

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watch was stopped as the last particle left the funnel. The time was recorded to the nearest 0.1 second. This same procedure was repeated five times for each sample and the average time in seconds was calculated.

The material was allowed to flow freely into the measuring cup without packing or tapping. Had the cup been tapped so as to increase the volume of granulation, theoretical values would have been approached more nearly, but in all probability the values would not have been more practical.

Test Granulations.—The samples consisted of the lactose granulation, "control fines," and lubricant. Granulations with 0, 10, 20, 30, 40, 45, 50, 55, 60, and 70% fines and no lubricant constituted one series of samples. The other series contained the same percentages of fines but lubricant was added in amounts of 1, 2, 3, 4 and 5 per cent. Based upon the theoretical investigation by Hoy (61), the fill ratio (Fr) and actual flow rate were determined. The fill ratio was the per cent of space occupied by the granules and was calculated as follows:

$$Fr = \frac{\text{Wt. of 100 ml. of granulation}}{\text{density} \times 100 \text{ ml.}}$$

The apparent volume was the space occupied by the granules and the void space between granules. The actual volume was the space occupied by the granules only. The apparent rate of flow was the volume of the cup (100 ml.) divided by the time in seconds. To obtain the actual number of milliliters that flowed per second, the apparent volume of flow was multiplied by the fill ratio. This actual flow rate was designated by the symbol Fa.

Results.—The data obtained as shown in Table I demonstrated that fines and lubricant do have a measurable effect upon the rate of flow with a general increase to a maximum followed by a decrease.

TABLE I.—ACTUAL VOLUME OF FLOW IN MILLILITERS PER SECOND (Fa)

Fines, %	Lubricant, %					
	0	1	2	3	4	5
0	2.32	2.58	2.85	2.74	2.80	2.81
10	2.64	2.83	2.88	3.01	3.09	3.08
20	2.71	2.97	3.05	3.15	3.22	3.19
30	3.03	3.23	3.34	3.50	3.47	3.49
40	3.69	3.38	3.49	3.59	3.62	3.68
45	3.92	3.57	3.65	3.73	3.73	3.83
50	3.41	3.55	3.72	3.80	3.81	3.89
55	3.42	3.76	3.78	3.72	3.80	3.95
60	3.32	3.67	3.73	3.69	3.85	3.86
70	3.35	3.58	3.43	3.49	3.61	3.56

Tablet Compression.—Samples from the same formulations as those used in flow rate determinations were used for compression in an attempt to correlate the former to the actual compression of tablets. The tablet machine was kept in adjustment to constantly deliver a tablet of 300 mg. in weight and a hardness of 8 as measured with a Strong, Cobb hardness tester. With this particular formulation tablets containing 0, 1, and 2% lubricant could not be compressed satisfactorily to render an acceptable product.

Evaluation of the Compressed Tablets.—The criteria of evaluation were hardness, weight variation, thickness, disintegration, and durability. These are usual standards with the exception of durability. The criteria in the case of durability was a measurement of the loss in weight of 20 tablets due to frictional erosion. The tablets were placed in glass vials and allowed to shake for ten minutes in an Arthur shaker. The weight of the 20 tablets was recorded before and after shaking and the loss in weight recorded in mg. (11, 54–56).

TABLE II.—FORMULA 16 WITH 3, 4, AND 5% TALC

Fines, %	Av. Wt., mg.	Av. Thickness, mm.	Hardness, Strong Cobb Units	Av. Disintegration, sec.	Durability Wt. Loss, mg.
			3%		
0	317	4.28	6.9	24.2	15
10	333	4.44	7.1	22.3	25
20	311	4.18	5.85	27.1	20
30	319	4.31	7.95	25.8	25
40	344	4.49	8.5	31.8	15
45	349	4.64	7.35	25.2	40
50	*	*	*	*	*
55	*	*	*	*	*
60	*	*	*	*	*
70	*	*	*	*	*
			4%		
0	321	4.17	10.2	46.3	15
10	314	4.25	5.9	26.7	25
20	504	4.21	5.0	22.7	35
30	314	4.29	5.7	24.5	40
40	332	4.43	6.35	26.9	40
45	344	4.50	6.65	28.2	20
50	338	4.53	5.25	23.6	45
55	322	4.39	5.15	22.4	20
60	332	4.45	6.2	25.8	25
70	331	4.44	5.7	24.2	30
			5%		
0	320	4.37	4.3	21.4	25
10	329	4.40	4.75	28.8	30
20	327	4.40	5.05	29.6	35
30	331	4.36	5.6	29.6	25
40	329	4.37	5.2	26.9	25
45	320	4.34	5.2	26.5	25
50	326	4.37	5.95	26.8	30
55	320	4.29	6.6	29.9	15
60	330	4.45	5.05	23.7	35
70	322	4.35	5.05	24.1	40

* Satisfactory tablets could not be compressed in this range using this formulation.

Statistical Analysis.—The data collected in this study lends itself to statistical analysis, and the experiments were designed to facilitate such an analysis.

To measure the uniformity of each sample the reciprocal of the coefficient of variation was used; this is termed the coefficient of uniformity (C. U.).

On these C. U.'s the following analysis was performed:

(*a*) For each per cent lubricant group an equation was fitted relating the per cent fines to uniformity.

$$(\bar{y}/s) = \text{C. U.} = b_0 + b_1 x_1 + b_2 x_1^2$$

b_0, b_1, and b_2 are constants determined from the data.

x_1 = per cent fines $\quad x_1^2$ = (per cent fines)^2

In actual calculations it was easier to code first the

per cent fines to reduce the size of the numbers. This coding was done by the formulas:

$$z_1 = (x_1 - \bar{x}_1);\ \bar{x}_1 = \text{mean per cent fines}$$
$$z_2 = (x_1 - \bar{x}_1)^2$$

The final form of the equation was then:

$$\text{C. U.} = b_0 + b_1 z_1 + b_2 z_2$$

(*b*) For each fit made, a test of goodness of fit was made by the use of the statistic R^2. R^2 is a measure of the strength of the relationship between per cent fines and uniformity. It may be thought of as varying from 0 to 1 where 0 is no relation and 1 is a perfect relation. It may also be thought of as $R^2 \times 100$ measuring the percentage of the variability in C. U.'s explainable by the different amounts of per cent fines, i.e., the "predictability" of uniformity knowing the per cent fines.

(*c*) Where R^2 was not larger than a value which could have occurred by chance (i.e., "nonsignificant") the conclusion is that there has not been a real relationship demonstrated.

(*d*) Three levels of "significance" were recognized: borderline, just short of the 5% level; significant, above the 5% value but short of the 1% value; and highly significant, exceeding the 1% value.

(*e*) Results were plotted for those measurements where R^2 was borderline or higher. For those results in which R^2 was less than borderline, the best estimate of the quantity is the mean value averaged over all per cent fines.

In the case of the durability and flow rate, uniformity was not the criterion, but the actual measurements themselves were related to the per cent fines in the same procedure as with the C. U.'s. Durability was measured as per cent weight loss, and flow rate was measured as ml. per second. See Figs. 1 and 2, and Table III for a summary of results.

DISCUSSION

As shown by the results in Table I the fines had a measurable and uniform effect upon the flow of a tablet granulation. The addition of fines increased the rate of flow to an optimum level, after which the rate began to decrease. A granulation of 100% fines will not flow freely. With addition of lubricant to a granulation an appreciable improvement in the rate of flow was noted. The addition of lubricant beyond 2% did not substantially improve the rate of flow. In this case an optimum amount was not the criterion sought, but rather the minimum amount that could be used with practical efficiency. The addition of lubricant increased the rate of flow

TABLE III.—R^2 (R = MULTIPLE CORRELATION COEFFICIENT) SHOWING RELATIONSHIP BETWEEN PER CENT FINES EXPRESSED AS THE C. U. AND PHYSICAL PROPERTIES OF TABLETS FOR VARIOUS PERCENTAGES OF LUBRICANT

C. U. = (g/s)	Lubricant, % 3	4	5
Weight	0.40	0.21	0.32
Thickness	0.86[a]	0.60[b]	0.78[a]
Hardness	0.15	0.67[a]	0.14
Disintegration	0.13	0.64[a]	0.35
Durability, wt. loss, %	0.24	0.02	0.01
Flow Rate, ml./sec.	0.96[a]	0.99[a]	0.92[a]

[a] Significance at the 1% level of probability.
[b] Borderline significance.
[c] Significance at the 5% level of probability.
Significance levels taken from Statistical Tables for Biological, Agricultural, and Medical Research by R. A. Fisher and F. Yates.

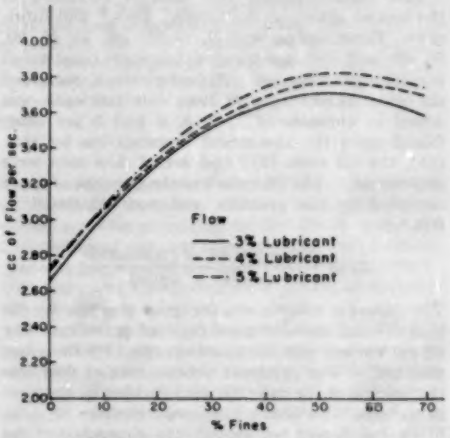

Fig. 1.—The predicted rate of flow.

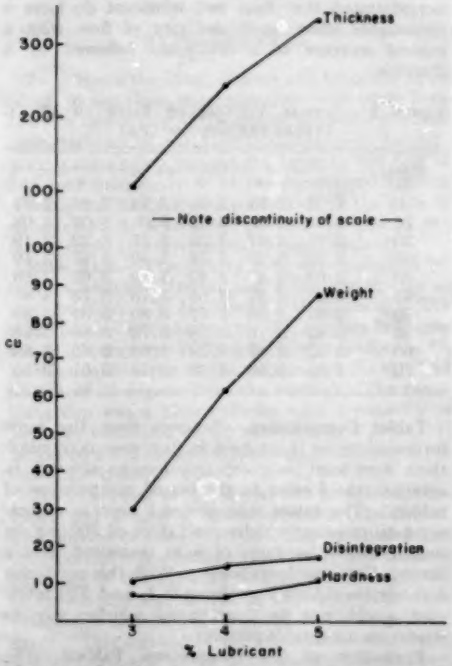

Fig. 2.—Total mean coefficient of uniformity.

to a maximum, and then it began to decrease. It seems logical to assume that beyond the maximum point the lubricant gives the effect of too many fines.

A combination of lubricant and fines appeared to have a synergistic action upon the rate of flow, as the rate increased when both fines and lubricant were added. Again it could be noted that a maximum point was reached, which was the optimum ratio of fines and lubricant. The maximum lay between 50 and 60% fines regardless of the concentration of the lubricant.

When fines and lubricant have such uniform effects upon the flow of the granulation, the rate of flow for a given formula is indicative as to its behavior.

In general the results as noted in Table II seem to follow the same pattern as the flow rate. The degree of consistency was not as great as in the case of the flow rate determinations. The lubricant was shown to be increasingly effective in those cases where tablets could not be compressed satisfactorily, but did not apply to preventing binding and sticking in the die. The measurement of lubricant by the ease of ejection alone is not completely satisfactory nor does its measurement by rate of flow suffice.

Table III shows the relationship between per cent fines and the physical properties of the tablets that were measured for given percentages of lubricant. Thickness and flow rate were the only factors that appeared to have a real relationship throughout. This does not mean that the data for which no real relationship was demonstrated are valueless, but it does indicate that the extent of the effects if present were of the same order of magnitude as the chance factors.

The tests that were conducted on durability were not considered in the coefficient of uniformity or as predicted values of the same, as its R^2 value was so low as to be considered chance effect.

The most striking results are the measurements based upon the rate of flow (Fig. 1). The correlation as shown by these results approach near perfection, 1. In this case the R value is greater than 0.9 in all instances.

It has been shown that a lubricant and fines will have a direct effect upon the physical characteristics of tablets in that the variation of these two factors will have a direct effect on the rate of flow. It can be said that of the contributing factors, the lubricant and fines will be responsible for more than 90% of the variation in flow rate.

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Correlation of Molecular Refraction with Structure in the Terpene Series*

By LUZ OLIVEROS-BELARDO† and PAUL J. JANNKE

Molecular refractions were determined for a number of terpenes and related compounds. Wherever optical exaltation occurred it was correlated with the structural configurations of the respective compounds. Optical isomers gave identical values while geometric isomers differed, with the *trans*-form giving the higher value. Isolated double bonds did not cause exaltation, but conjugated double bonds definitely increased the molecular refraction. This was particularly marked when an exocyclic bond was conjugated with one within the cycle. A double bond in conjugation with a three membered cycle gave higher exaltation than one in conjugation with a four membered cycle. The unique influence of the isopropyl group on the cyclic double bond nearest to it results in a marked change in molecular refraction.

TEXTBOOKS ON qualitative organic analysis mention molecular refraction as a valuable aid in the identification of organic compounds, yet this physical constant is seldom reported in the literature. Guenther (1) states that molecular refraction is a valuable constant frequently employed in determining the identity of isolates from the essential oils. However, in his own treatise, which is by far the most comprehensive work published on the volatile oils to date, molecular refraction is seldom listed among the physical constants of the terpenes and terpenoids.

Being replete with isomerism of all kinds, the terpenes present unusual problems in identification. This study was undertaken to disclose correlations which could be drawn between structural features of the isomers and variations in the basic molecular refraction.

DISCUSSION

Molecular refraction is fundamentally the sum of the individual atomic refractions. These values, however, show variation based on generic differences as is illustrated by oxygen in the hydroxyl, carbonyl, carboxyl, and ether types of linkages. The simple sum of the appropriate values may not agree with experimental results because of exaltation or depression resulting from inherent structural characteristics, such as a double bond conjugated with a cycle, a side chain or another double bond. Among isomers, subtle structural differences may account for wide variations in molecular refractions, and these variations when determined experimentally can be interpreted in terms of distinctive structural habits. Correlation of such data should aid materially in the rapid identification of known terpenes and in projecting the structure of terpenes not yet known.

Experimental data compiled in this work was applied to the n^2 formula of Lorentz and Lorenz (2) which, it is claimed, is independent of temperature, pressure, and state of aggregation. In calculating the theoretical molecular refractions, the atomic refractions for the D-line determined by Brühl and Conrady (3) were found to give the most accurate results.

Understandably, the samples of terpenes and related compounds used in this study were necessarily of the highest purity attainable. Pure compounds of this type are not available commercially, therefore they were either isolated from the essential oils of high quality or were donated by laboratories of high repute.

Generally, samples were distilled at reduced pressure immediately prior to use. For the larger samples, a Todd precise fractionation assembly was used, at various reflux ratios. When the volumes of samples were of the order of 2.5 ml. and less, small scale fractionating equipment[1] was employed. Isolates were held under nitrogen in tightly closed containers. They were maintained in a constant temperature bath for about twelve hours prior to being used in measurements. The prisms of the refractometer[2] were maintained at 25° by means of a circulating water-temperature regulator. Whenever possible, specific gravities were determined in a 10-ml. Geissler pycnometer, for greater accuracy. When only small volumes of samples were available, Sprengel-Ostwald type pycnometers with capacities ranging from 0.1 to 5.0 ml. were employed. These, too, were maintained at a constant temperature by keeping them in a closed container which was immersed in the water bath.

The data which are recorded were checked repeatedly in order to be assured of their accuracy. The only substance which did not behave consistently was *d*-limonene. That which was produced by the fractionation of sweet orange oil (cold-pressed), and repeatedly distilled from metallic sodium, appeared to be identical with a sample furnished by the Naval

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[1] Metro Industries, Long Island City, N. Y.

[2] Bausch and Lomb Optical Co., Type 33-45-50.

Stores Station. On the other hand, *d*-limonene isolated from lemon oil boiled at only 0.1° less than the *d*-limonene from orange oil, yet the differences between their specific gravities, refractive indices, and optical rotations were very great. Based on the low rotation of the *d*-limonene from lemon oil, it might be concluded that some *l*-limonene could be present, however *l*-limonene has never been reported to occur in citrus oils. The alternative explanation is that in addition to *d*-limonene of conventional configuration lemon oil may contain the *d*- and *l*-forms of the strainless ring isomers (the chair and the boat forms) whose angular rotation, depending upon their proportions, could depress that of *d*-limonene of planar configuration. It is of significance to note that the refractive index (1.4725) reported for *d*-limonene by some investigators (4) was attained only after the limonene from lemon oil stood for sixteen days under ordinary conditions of storage.

EXPERIMENTAL

Samples of hydrocarbons obtained from the various sources[3] were carefully prepared for physical measurements (see Discussion) which were applied in the calculation of molecular refractions (Table I).

CONCLUSIONS

An analysis of the data given in Table I affords the following conclusions.

1. Based on the figures referred to by past investigators as to the range of normal and anomalous refractions,[4] the results of this investigation show that normal molecular refraction was shown by trans-*p*-menthane, cis-*p*-menthane, 3-*p*-menthene, limonene, dipentene, terpinolene, *p*-cymene, and *α*-pinene. Those that gave exaltation were 3,8-*p*-menthadiene, *α*-terpinene, *d*-*α*-phellandrene, *β*-pinene, and sabinene.

2. The identical molecular refraction obtained for limonene from oil of orange (IV, Table I) and dipentene (V, Table I) shows that optical isomerism introduces no difference in molecular refraction.

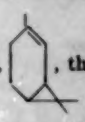
3. It will be noticed that even within the group of isomeric compounds showing normal molecular refraction, the effect of structural variation is demonstrated, in that there exist in some of them a difference in degree of normal refraction. Thus, trans-*p*-menthane (I, Table I) gave a higher value than its isomer, cis-*p*-menthane (II, Table I). The foregoing results agree with the findings of Brühl who showed that optical isomers give the same molecular refraction while geometric isomers differ, the trans-form giving the higher value.

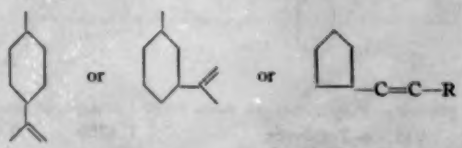
4. Since the molecular refraction of limonene, dipentene, and terpinolene (IV, V, and VIII, Table I) fall within the range of normal values, the isolated double bonds in each of these compounds did not cause optical anomaly.

5. Of the bicyclic terpenes used in this investigation, *β*-pinene and sabinene showed marked exaltations. *α*-Pinene gave the highest refraction among those that gave normal refractions.

6. Conjugated double bonds definitely raised molecular refraction. 3,8-*p*-Menthadiene (VI, Table I), *α*-terpinene (VII, Table I), and *d*-*α*-pheliandrene (IX, Table I) all showed optical exaltation. Of these hydrocarbons, 3,8-*p*-menthadiene gave the highest exaltation, indicating that an exocyclic double bond conjugation with a double bond inside the ring produces high exaltation.

p-Cymene (X, Table I), with its three double bonds in conjugation, became an exception. The sudden drop from the marked exaltation of *α*-terpinene to the normal refraction of *p*-cymene is consistent with reports that the benzene ring does not show optical exaltation.[5]

7. The exaltation of *β*-pinene and sabinene and the almost low exaltation of *α*-pinene show that a double bond conjugatiug with a cycle could cause exaltation. From the result obtained for sabinene, and from reports in literature (5) for Δ^4 carene,  , the location of the cycle that is in conjugation with the double bond is immaterial, i. e., it may be an inner or an outer cycle. As to whether or not this property holds true only for bicyclic compounds cannot be held conclusive until measurements are made for compounds having the following types of configuration:



where a double bond lies in conjugation with a cycle in a monocyclic compound.

8. Comparing the exaltations obtained for

[3] The authors gratefully acknowledge the receipt of samples of hydrocarbons for this study. The Hercules Powder Co., Wilmington, Del., kindly furnished cis-*p*-menthane, 3-*p*-menthene, 3,8-*p*-menthadiene, *α*-terpinene and terpinolene. The Naval Stores, U. S. D. A., Olustee, Fla., kindly supplied trans-*p*-menthane, *d*-limonene, and *α*-pinene.

[4] A difference of +0.03 to +0.26 was considered "normal" refraction; +0.34, "little exaltation," +0.5, "exaltation"; and above +0.5, "marked exaltation."

[5] Measurements for purified toluene and purified benzene were made as a side line to this work. It was observed that both of these hydrocarbons did not show exaltation.

TABLE I.—STRUCTURAL CONFIGURATION AND MOLECULAR REFRACTION

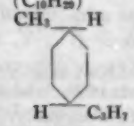
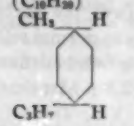
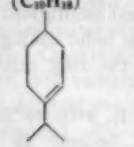
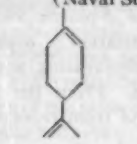
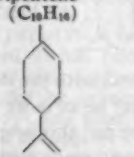
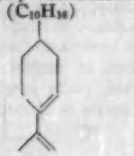
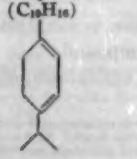
Compounds	n_D^{25}	d_{25}^{25}	Molecular Refraction		
			Calcd.	Found	Difference
I. Trans-*p*-menthane ($C_{10}H_{20}$) 	1.4364	0.7962	46.03	46.08	+0.05
II. Cis-*p*-menthane ($C_{10}H_{20}$) 	1.4412	0.8085	46.03	45.81	−0.22
III. 3-*p*-Menthene ($C_{10}H_{18}$) 	1.4501	0.8109	45.64	45.81	+0.17
IV. *d*-Limonene ($C_{10}H_{16}$) 					
From Lemon Oil[a]	1.4718	0.8492	45.24	44.90	−0.34
From Orange Oil[b]	1.4706	0.8414	45.24	45.20	−0.04
From Naval Stores[c]	1.4709	0.8413	45.24	45.24	−0.00
V. Dipentene ($C_{10}H_{16}$) 	1.4708	0.8420	45.24	45.20	−0.04
VI. 3,8-*p*-Menthadiene ($C_{10}H_{18}$) 	1.4902	0.8550	45.24	46.08	+0.84
VII. α-Terpinene ($C_{10}H_{16}$) 	1.4759	0.8373	45.24	45.86	+0.62

TABLE I (*Continued*)

	Compounds	n_D^{25}	d_{25}^{25}	Molecular Refraction		
				Calcd.	Found	Difference
VIII.	Terpinolene ($C_{10}H_{16}$)	1.4877	0.8632	45.24	45.42	+0.18
IX.	*d*-α-Phellandrene[d] ($C_{10}H_{16}$)	1.4691	0.8323	45.24	45.58	+0.34
X.	*p*-Cymene[e] ($C_{10}H_{14}$)	1.4878	0.8584	44.84	45.02	+0.18
XI.	α-Pinene ($C_{10}H_{16}$)	1.4636	0.8577	43.53	43.79	+0.26
XII.	β-Pinene[f] ($C_{10}H_{16}$)	1.4733	0.8650	43.53	44.19	+0.66
XIII.	Sabinene[g] ($C_{10}H_{16}$)	1.4641	0.8424	43.53	44.61	+1.08

[a] Limonene fraction b. p. 66.3°, at 15 mm.; $[\alpha]_D^{25}$ + 86°36′.
[b] Limonene fraction b. p. 66.4°, at 15 mm.; $[\alpha]_D^{25}$ + 122°24′.
[c] B. p. 66.4°, at 15 mm.; $[\alpha]_D^{25}$ + 122° 24′.
[d] Distilled from Manila elemi oil, b. p. 61.0°; +117°2′.
[e] Commercial terpene-free *p*-cymene distilled at 15 mm. yielded fraction 53.5–54.0°, which was redistilled from metallic sodium at 760 mm., b. p. 174.0°.
[f] From Canadian fir oil, b. p. 165–165.5°, at 760 mm.
[g] From savin oil, b. p. 66.5°, at 30 mm.

β-pinene (XII, Table I) and sabinene (XIII, Table I), and taking into consideration the reported exaltation for Δ^4 carene, it appears that an ethenoid linkage conjugating with a three-membered ring produces exaltation higher than that produced by conjugation with a four-membered ring.

9. The exalting effect of an ethylenic bond

outside but attached to the ring was demonstrated in terpinolene (VIII, Table I), β-pinene (XII, Table I), and sabinene (XIII, Table I). The exaltation due to conjugation between an ethenoid linkage and a cycle was already explained in one of the preceding paragraphs. However, α-pinene, with its double bond conjugating with a cyclobutane ring does not show this effect because its difference of 0.256, although the highest obtained among the normal refractions in this work, does not come up within the range of what can be called true exaltation. But in shifting the double bond to the outer part of the ring, as in β-pinene, there is produced marked exaltation. Therefore, the position of the double bond, more than the conjugation between double bond and cycle, seems to be responsible for the marked exaltation. The observed molecular refraction for terpinolene supports this observation. While the molecular refraction for terpinolene lies within the normal values, it is far above those for dienes where an exocyclic ethenoid linkage is removed from the ring as in limonene and dipentene.

The high exaltation of sabinene could probably be interpreted as a function of the presence of two highly influential conditions for exaltation; namely, the ethenoid bond attached to the nucleus, and the same, conjugating with a three-membered ring. Further studies along this line would have been worthwhile had there been some α-thujene, , available for comparison and verification.

10. The isopropyl group seems to exert an exalting influence on the cyclic double bond nearest to it. Although the measurements for benzene and toluene were done primarily for verification of the nonexalting effect of the benzene ring, the results of the analysis tend to show the effect of the isopropyl group on molecular refraction in the *p*-menthene series. Benzene gave a difference of −0.248, and toluene −0.1268, or a difference of −0.1212 between them. But from toluene to *p*-cymene, a sudden rise of +0.3077 difference was noted. Since the essential structural difference between toluene and *p*-cymene lies in the isopropyl radical, it is evident that the sudden rise in difference must have been brought about either by the isopropyl group *per se*, or by a combined effect of the presence of the group and its influence on the neighboring double bond.

Results of measurements for α-phellandrene and α-terpinene lend a strong support to the combined effect, since both of them contain the isopropyl group. They also have a system of conjugated double bonds inside the cycle. The 1-2 position of one double bond is common to them. The only apparent difference that could create the wide gap between their molecular refractions is the position of their respective second double bond; that of α-terpinene being nearest to the isopropyl group. By the same token, the marked exaltation of 3,8-*p*-menthadiene may be due, not only to the system of conjugation occurring between an exocyclic and cyclic bonds, but also to what may be a unique influence of the isopropyl group on the double bond in position 3-4.

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Studies on the Effects of Ephedrine in the Presence of Cocaine*

By PHILLIP V. HAMMOND†

The pressure response of ephedrine was found to be reduced in anesthetized dogs by the previous administration of cocaine, as has been reported in earlier works. It was determined that this reduction was not due to tachyphylaxis. A definite relationship between dosage of cocaine, time allowed for the animal to become cocainized, and a lessening to the response of ephedrine were observed. Similar effects were also ascertained in studies on spleen volume. The action of the two drugs on cardiac output was determined by means of the radioactive isotope dilution method using I^{131}.

IT HAS BEEN OUR EXPERIENCE to note that ephedrine in the presence of cocaine does not produce a rise in the blood pressure of the anesthetized dog within ranges usually observed with a given dose of ephedrine. Indeed on one or two occasions the rise in blood pressure induced by ephedrine in the cocainized animal was so slight that it was insignificant.

At one time ephedrine was thought to have a mechanism of action similar to epinephrine (1), i. e., acting through the same receptor mechanism. However, this does not explain the many unrelated actions such as tachyphylaxis, central nervous system stimulation, prolonged action, and desensitization by cocaine.

In 1928 Chen (2), using a small dose of cocaine (10 mg.), reported the effects of cocaine on the pressor action of ephedrine. Chen reported that the rises of blood pressure produced were within normal ranges. However, Tainter (3) in 1929 reported that cocainization of cats and dogs prevented or greatly decreased the pressor, cardiac, and respiratory responses to ephedrine and simultaneously sensitized the pressor and cardiac responses to epinephrine. Tainter also reported that he obtained the desensitization to ephedrine and also the sensitization to epinephrine regardless of the type of anesthesia and also in the absence of anesthesia.

In 1933 Holck (4) explained a method for demonstrating the difference between ephedrine and epinephrine blood pressure response after cocaine. In 1953 Shinyo (5) reported that cocaine was rather synergistic with epinephrine but to other sympathomimetic amines it was rather antagonistic. Despite the effects of many investigators who have contributed to the vast amount of literature on this drug, the exact mechanism of action has not been definitely established. This paper, based largely on pilot experiments, is concerned with further elucidation of the mechanism of action of ephedrine on the cardiovascular system in the presence of cocaine.

EXPERIMENTAL

Pressure Response.—Throughout the pressor experiments a total of twelve mongrel dogs of both sexes were used. The trachea and the carotid artery were isolated and cannulated. Blood pressure was recorded by means of a mercury manometer. Respiratory changes were recorded by means of a tambour. A 4% solution of cocaine hydrochloride in varying doses was injected subcutaneously and a 1% solution of ephedrine hydrochloride in doses of 0.3 mg./Kg. was injected into the femoral veins of dogs under sodium pentobarbital anesthesia (30 mg./Kg.). The procedure was to ascertain the normal blood pressure after the animal had become stabilized, inject ephedrine, and after a suitable interval of at least forty-five minutes and the blood pressure had returned to normal, cocaine was injected. Twelve minutes were allowed for the animal to become cocainized. At the termination of the cocanization period a second injection of ephedrine was administered. This procedure was repeated three or four times. Electrocardiographic changes were taken with a Sandborn Viso-Cardiette using standard limb leads.

Isolated Heart.—Cocaine and ephedrine are known to be antagonistic on the heart. Several experiments were carried out on the isolated rabbit heart using the Langendorff and Anderson pump (6, 7). The purpose was to observe whether or not this antagonism could be demonstrated in the isolated heart preparation. The animal was sacrificed by a sharp blow on the back of the neck. The heart was quickly removed and placed into the chamber. Here it was oxygenated and perfused with a modified Ringer's solution at a constant temperature of 37.5°. After the normal amplitude, the normal rate of contraction, and the perfusion rate had been determined, the two drugs were perfused through the preparation at definite intervals. Cocaine was introduced in doses of 4 mg. whereas ephedrine was employed in doses ranging from 1.5 mg. to 15 mg.

Splenic Volume Change.—Ephedrine in the presence of cocaine was tested on splenic volume changes by opening the abdomen of small anesthetized dogs,

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exposing the spleen and placing it in an oncometer. The oncometer was made airtight by covering the opening with gauze which had been impregnated with petrolatum. Volume changes were registered by a tambour and made to record on a kymograph. Cocaine in doses of 15 mg./Kg. was injected subcutaneously. Ephedrine in doses of 0.3 mg./Kg. was injected intravenously.

Cardiac Output.—Cardiac output studies were conducted by means of the radioactive isotope dilution method (8) using iodinated (I^{131}) human-serum albumin. Twenty cubic centimeters of Lugol's solution were administered orally to prevent the thyroid from picking up the radioactive iodine. Sodium pentobarbital 30 mg./Kg. was used as the anesthetic agent. The jugular vein and femoral artery were isolated and cannulae and Cournand needle were inserted. Iodinated (I^{131}) human-serum albumin was injected in doses of 30 microcuries into the jugular vein. The dilution technique employed was to dilute 1 cc. of radioactive iodinated human-serum albumin to 5 cc. with normal saline. Of this dilution, 0.1 cc. was diluted to 10 cc. One cubic centimeter of the aliquot was used as a control. The volume of the dose was 4.4 cc. The dilution curve was obtained by plotting the radioactivity of the blood flowing over the detector as a function of time.

RESULTS

Pressor Response.—In all experiments, and with doses of cocaine as little as 4 mg./Kg., the pressor response to ephedrine was reduced. Represented values of this diminution are presented in Fig. 1. The reduced pressor response of ephedrine in the cocainized animal was found to increase with increased doses of cocaine against a standard dose of ephedrine. A dose of 30 mg./Kg. of cocaine prevented 80.65% of the pressor response to cocaine. This was greater than that obtained by Hamet (9). Hamet reported that ephedrine alone increased 129%, and that ephedrine after cocaine only increased the blood pressure on the average of 71%, a reduction of 58% of the normal pressor response. In one experiment ephedrine did not increase the blood pressure in the cocainized animal. However, these results were not reproducible. In one experiment cocaine was injected into the animal prior to the administration of ephedrine. This was followed by a dose of ephedrine at the end of twelve minutes. In this instance the inhibition of the pressor effect which occurred when a single dose of ephedrine was administered was less than in experiments when cocaine followed the first dose of ephedrine and subsequent doses of ephedrine were administered at definite intervals. For example at a dose of 15 mg./Kg. cocaine alone produced a 20% diminution in pressor response to ephedrine; whereas cocaine at a dose of 15 mg./Kg. produced a 78% diminution in pressor response to ephedrine when administered to an animal who had received one dose of ephedrine. An interval of one hour or more between doses of ephedrine (0.3 mg./Kg.) prevented tachyphylaxis.

Electrocardiographic changes revealed that immediately after the first dose of ephedrine there was apparently no change in heart rate. However, five minutes after the administration of ephedrine there was an average increase in heart rate of 50%. Because of the increase the *T* and *P* waves became closer. The change in voltage or amplitude was insignificant, and the *S* wave became depressed. Five minutes after the administration of cocaine, the heart rate remained the same, and the amplitude remained the same; however, the *S* wave became more depressed. Ten minutes after the administration of cocaine, the *S* wave appeared further depressed; the heart rate remained the same, and the amplitude remained the same; however, the *S* wave became more depressed. The maximum effect after the administration of cocaine revealed that the *S* wave was further depressed; the heart rate remained the same; and the *T* wave appeared more prominent.

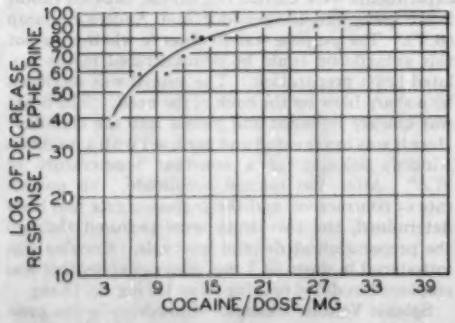

Fig. 1—Diminution of pressor response to ephedrine in the cocainized animal.

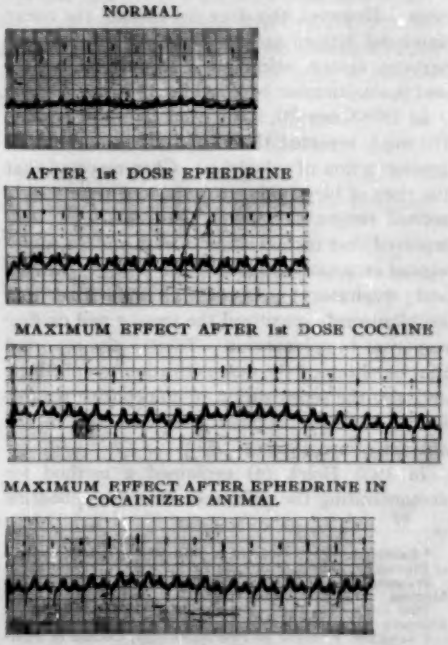
NORMAL
AFTER 1st DOSE EPHEDRINE
MAXIMUM EFFECT AFTER 1st DOSE COCAINE
MAXIMUM EFFECT AFTER EPHEDRINE IN COCAINIZED ANIMAL

Fig. 2—Cardiographic changes of ephedrine in the presence of cocaine.

Immediately after the administration of ephedrine in the cocainized animal, the heart rate remained the same; the *S* wave was still depressed; and the *T* wave became less prominent than when seen following cocaine. Five minutes after the second dose of ephedrine in the cocainized animal, the amplitude and the rate remained the same; however, the *S* wave depression was somewhat less.

The maximum effect of ephedrine in the cocainized animal revealed a decrease in heart rate, prominent depression of the *S* wave, a slight fall in voltage or amplitude, and no changes in rhythm.

Isolated Rabbit Heart.—Ephedrine did not afford protection against cocaine. However, with increased doses of ephedrine, the response to the depression of cocaine was somewhat lessened. The toxic levels of ephedrine, however, were soon reached, which levels resulted in a marked decrease in rate and response similar to that induced by cocaine.

Splenic Volume Changes.—As seen in Fig. 3, ephedrine alone produced not only a marked increase in blood pressure, but also a similar increase in contraction of the spleen. Cocaine produced a slight degree of contraction; however, ephedrine in the presence of cocaine produced no significant change in splenic contraction.

Cardiac Output.—Cardiac output studies are still in progress. However, there appears to be a definite trend which may be typified by the following experiment (see Table I).

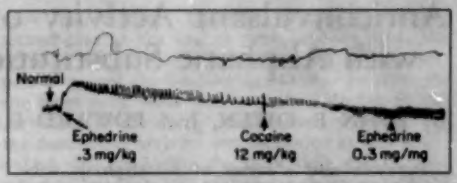

Fig. 3—Splenic contractions.

prior to the administration of cocaine, the *S* wave appeared depressed and the *T* wave appeared more prominent. The maximum effect of ephedrine in the cocainized animal showed a substantial drop in heart rate from that seen after ephedrine alone; this was accompanied by a slight fall in amplitude. There was no change in rhythm; however, there was a marked depression of the *S* wave. This latter finding is in keeping with the increase in cardiac output.

3. Splenic volume changes (i. e., contraction) accompanied the increase in blood pressure induced by ephedrine. This splenic contraction was abolished by the prior administration of cocaine.

4. Ephedrine increases the cardiac output.

TABLE I

	Normal	Ephedrine, 0.3 mg./Kg.	Ephedrine 0.3 mg./Kg. in the Cocainized Animal
Cardiac Output	3.63 L./Min.	5.77 L./Min.	5.33 L./Min.
Cardiac Index	4.5 L./Min./M²	7.2 L./Min./M²	6.67 L./Min./M²
Total Peripheral Resistance, Gm./dynes/cm.³	2,564	1,605	1,738
Heart Rate	170	180	175

From Table I, it can readily be seen that ephedrine substantially produces an increase in cardiac output. However, the reduction in cardiac output induced by ephedrine in the presence of cocaine was not so great as expected. Further, it was somewhat surprising that the total peripheral resistance was lessened. Also the increase in heart rate was not so great as that revealed by the electrocardiographic method. More cardiac output studies are planned.

SUMMARY AND CONCLUSIONS

1. Experiments have been described demonstrating reduced response of cocainized animals to standard doses of ephedrine. Under proper conditions this reduction amounted to as much as 90 per cent. It was determined that tachyphylaxis was not responsible for the diminution in response to ephedrine in the cocainized animal after an interval of one hour or longer.

2. Electrocardiographic studies revealed that ten minutes after the administration of cocaine, the heart rate remained very much the same as The reduction produced by the prior administration of cocaine is not great.

5. The increase in cardiac output induced by ephedrine is due mainly to the increase stroke volume rather than to an increase in heart rate.

6. Throughout these studies it has been clearly demonstrated that the effects of ephedrine are lessened by the presence of cocaine. Exactly how this effect is accomplished has not been ascertained.

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Anticonvulsant Activity of Pyrazole and of Pyrazole with Aliphatic Substitutions at the Three Position*

By JOHN E. OWEN, Jr.,† EDWARD E. SWANSON,‡ and DONALD B. MEYERS§

Pyrazole and a series of 3-*n*-aliphatic substituted pyrazoles have been shown to have anticonvulsant activity in rats and mice. In this study, extension of the length of the aliphatic chain up to four carbons increased the acute toxicity and anticonvulsant activity of these compounds. Further lengthening of the substituents decreased the toxicity and activity. The compounds, as anticonvulsants, were found to be more active in rats than in mice.

AMONG A SERIES of nitrogen-containing five-membered heterocycles investigated for central nervous system activity by Gibson, *et al.* (1), some substituted pyrazoles were found to protect rats from convulsions induced by electroshock and pentylenetetrazol (Metrazol). This finding has added another type of compound to the group of drugs classified as anticonvulsants. A survey of the literature has revealed relatively little information on the pharmacology of pyrazole compounds. Those on which data are available have been reported to exert a variety of actions, including central nervous system stimulation and depression, diuresis, and some musculotropic action on smooth muscle. The depression has been characterized by varying degrees of sedation, analgesia, and antipyresis. However, Gibson, *et al.* (1), appear to have been the first to report on the anticonvulsant activity of pyrazoles.

Because certain of these pyrazoles with substitutions at the 3-position were found to possess significant anticonvulsant activity, further study of a homologous series substituted at that position seemed indicated. The investigation reported here was limited to an examination of the influence of straight-chain aliphatic substitutions at the 3-position on the acute toxicity and anticonvulsant properties of pyrazole.

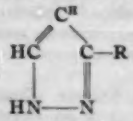
EXPERIMENTAL

Materials and Preparation.—The compounds studied were: pyrazole, 3-methylpyrazole, 3-*n*-propylpyrazole, 3-*n*-butylpyrazole, 3-*n*-amylpyrazole, and 3-*n*-nonylpyrazole. The compounds were prepared by the following general reaction:[1]

$$RCOCH_3 + H_5C_2OCHO \xrightarrow{NaH} RCOCH_2CHO$$

$$RCOCH_2CHO + H_2NNH_2.HCl \xrightarrow{\text{ether}}$$

R = aliphatic substituent



+ NaCl

These compounds, with the exception of pyrazole, are clear, yellow oils. Pyrazole is a white crystalline substance which melts at 70°. All compounds were relatively insoluble in water.

Methods.—The compounds were administered orally as gum acacia emulsions. Rats of both sexes of a Wistar derived strain, weighing 90–140 Gm.; and mice of both sexes of the Bittner-A strain, weighing 15–30 Gm., were employed for the assays.

Acute Toxicity.—The median lethal dose (LD_{50}) with standard error (S. E.) was determined for each compound in both the rats and the mice. The animals were observed for lethal effects over a period of seventy-two hours.

Anticonvulsant Activity.—Anticonvulsant activity was evaluated by the maximal electroshock seizure test (M. E. S.) of Toman, *et al.* (2), and by the subcutaneous Metrazol seizure test described by Swinyard (3). A dose response curve of anticonvulsant activity at a predetermined time of peak activity was established for each compound on each of the procedures. The median lethal and median effective (ED_{50}) doses with standard errors were computed by the Bliss method (4).

RESULTS

The data obtained from the assays of these pyrazole compounds have shown that pyrazole itself has anticonvulsant activity in rats and mice and that normal aliphatic substitutions at the 3-position quantitatively altered this activity (Tables I, II, and III).

Lengthening of the chain up to four carbon atoms increased both toxicity and anticonvulsant activity. When the chains were further increased in length to the amyl and nonyl compounds, the activity and toxicity decreased. There was one exception: 3-methylpyrazole was less active than pyrazole in rats on the subcutaneous Metrazol® test. A comparison of the therapeutic indexes (T. I.), calculated as LD_{50}/ED_{50}, of the three most potent of the compounds on each type of assay in both species, showed

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Abstracted from a thesis submitted to the Graduate School of Butler University by John E. Owen, Jr., in partial fulfillment of the requirements for the degree of Master of Science.
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‡ Present address: College of Pharmacy, Butler University, Indianapolis 7, Ind.
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[1] The compounds used in this study were prepared by Dr. Nelson R. Easton of the Organic Chemistry Division of The Lilly Research Laboratories.

the 3-*n*-butylpyrazole to have the optimal activity and safety margin on all procedures except the subcutaneous Metrazol test in mice. The 3-*n*-butyl compound appeared to be the most active member of the series but the 3-*n*-propylpyrazole had a slightly better T. I.

At the doses used to determine acute toxicities, the pattern of depression produced in the rats and the mice was the same for all of the compounds. The animals became ataxic with general muscle weakness followed by loss of righting reflexes and slowed respirations. They became comatose with pronounced cyanosis and death resulted from respiratory failure.

The onset of peak activity (Table IV) was more rapid and the duration of peak activity shorter in mice than in rats. However, in rats, it was observed that at the end of peak activity time, the activity fell off very rapidly with all of the compounds except the 3-*n*-nonylpyrazole. In mice, for the most part, there was a gradual decline in the activity after the end of peak action. In Table IV the duration of activity was estimated as that interval of time, including the peak time duration, at which no less than 25% of the animals were protected from convulsions.

All compounds proved more active as anticonvulsants in rats than in mice. The 3-*n*-propylpyrazole, and 3-*n*-amylpyrazole were more toxic in rats than in mice. The T. I.'s were shown to be more favorable in rats than in mice.

TABLE I.—LD_{50} ± S. E. OF PYRAZOLE AND 3-ALIPHATIC SUBSTITUTED PYRAZOLES IN RATS AND MICE

Substituent	LD_{50} ± S.E., mg./Kg. Rats	Mice
Hydrogen	1734.0 ± 109.2	1456.0 ± 96.1
Methyl	1312.0 ± 78.7	885.5 ± 59.3
Propyl	615.5 ± 52.3	778.0 ± 38.1
Butyl	509.3 ± 25.5	543.7 ± 24.5
Amyl	522.9 ± 41.3	637.7 ± 27.4
Nonyl	2695.0 ± 207.5	1078.0 ± 87.3

DISCUSSION

The compounds were tested in both rats and mice to determine whether a species difference existed. Swinyard, *et al.* (5), considered that either animal is suitable for this type of testing but that it is desirable for a more definitive comparison to use both species. Furthermore, unless the compounds being tested are chemically related to agents known to have antiepileptic effects, these assays may be seriously limited in predicting any clinical efficacy unless at least two species are used. In this evaluation of 3-*n*-aliphatic substituted pyrazoles as anticonvulsants, the qualitative results were similar for

TABLE II.—ED_{50} ± S. E. AND T. I. OF PYRAZOLE AND 3-ALIPHATIC SUBSTITUTED PYRAZOLES AGAINST M.E.S.

Substituent	Rats ED_{50} ± S.E.	Rats T. I.	Mice ED_{50} ± S.E.	Mice T. I.
Hydrogen	89.32 ± 8.22	19.48	284.10 ± 11.60	5.13
Methyl	66.99 ± 6.79	19.88	173.90 ± 6.80	5.12
Propyl	32.86 ± 3.00	19.22	101.50 ± 4.50	7.70
Butyl	14.70 ± 2.06	36.35	66.99 ± 4.42	8.23
Amyl	37.33 ± 3.88	14.11	79.49 ± 5.17	8.06
Nonyl	48.09 ± 4.81	56.15	86.24 ± 8.50	12.53

TABLE III.—ED_{50} ± S. E. AND T. I. OF PYRAZOLE AND 3-ALIPHATIC SUBSTITUTED PYRAZOLES AGAINST METRAZOL INDUCED SEIZURES

Substituent	Rats ED_{50} ± S. E.	Rats T. I.	Mice ED_{50} ± S. E.	Mice T. I.
Hydrogen	98.47 ± 11.52	17.70	377.6 ± 19.2	3.89
Methyl	131.80 ± 9.70	10.84	236.2 ± 27.6	3.75
Propyl	76.58 ± 13.02	8.09	138.7 ± 8.6	5.64
Butyl	38.10 ± 6.48	13.40	109.5 ± 7.7	4.98
Amyl	71.93 ± 7.34	7.35	226.0 ± 22.7	2.82
Nonyl	141.90 ± 20.60	19.11	327.3 ± 17.3	3.30

TABLE IV.—PEAK TIME AND DURATION OF ANTICONVULSANT ACTIVITY OF PYRAZOLE AND 3-ALIPHATIC SUBSTITUTED PYRAZOLES

Substituent	Rats Dose, mg./Kg.	Rats Peak Time, min.	Rats Duration, min.	Mice Dose mg./Kg.	Mice Peak Time, min.	Mice Duration, min.
Hydrogen	70	300–360	*	160	240	*
Methyl	80	300–360	*	250	90	300
Propyl	40	60–120	*	125	15–30	200
Butyl	25	30–120	*	80	15–45	75
Amyl	40	30–120	*	80	15–60	*
Nonyl	50	60–120	180	100	15–45	90

* The drug activity at onset of action rose very rapidly to the peak and fell off rapidly after peak time.

both species. A quantitative difference was observed, however, since the compounds were more active in rats than in mice.

The two tests used in this investigation readily lend themselves to the evaluation of a large number of compounds (6), the usually given end points of the tests are well defined and the results are reproducible. Drugs found to be clinically effective against grand mal epilepsy will as a rule appear active in the maximal electroshock seizure test. Those agents which are active against petit mal seizures will protect animals given Metrazol subcutaneously in threshold convulsive doses. Some drugs, such as the barbiturate antiepileptics, will show a good cross activity between the two procedures and this has been confirmed clinically.

A new group of compounds with anticonvulsant activity in rats and mice has been presented. This group represents another departure from the earlier suggestions that the more potent anticonvulsant agents have certain basic similarities in the structural nuclei (7, 8). The investigation reported here has shown 3-*n*-aliphatic substituted pyrazole compounds active at dose ranges comparable to the activity found in experimental animals with some of the commonly used antiepileptic drugs. In spite of the short duration of activity in experimental animals, the potency and T. I.'s of some of these pyrazoles suggest their suitability for clinical investigation. In view of these findings, further studies of other substitutions on pyrazole should prove fruitful in revealing more interesting compounds with neuropharmacological activity.

SUMMARY

1. Pyrazole and a series of 3-*n*-aliphatic substituted pyrazoles have been shown to possess anticonvulsant activity.

2. The optimal length of the aliphatic substitution for maximum anticonvulsant activity in both rats and mice appears to be a chain of four carbons.

3. The compounds were more active in rats than in mice as anticonvulsants.

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Nitro-organophosphorus Compounds I.*

Phosphite and Phosphate Esters of 2-Nitro-2-methylpropanol-1

By WILLIAM SHELVER, M. I. BLAKE, and C. E. MILLER

A laboratory method has been developed for the preparation of the phosphite and phosphate esters of 2-nitro-2-methylpropanol-1. Permanganate oxidation of the phosphite yielded the phosphate.

PREVIOUS PUBLICATIONS (1–3) from this laboratory have reported the preparation of various nitroalkyl compounds for screening purposes in connection with our cancer research program. A further pharmacological study of one of these compounds, bis(2-nitro-2-methylpropyl)-sulfite has been reported (4). Within recent months certain phosphorus derivatives such as triethylene phosphoramide (5), have been reported as showing efficacy against certain neuroblastomas and malignant melanomas. In view of the activity of this phosphorus compound it was believed worthwhile to extend the study of aliphatic nitrocompounds to include the phosphite and phosphate esters. Such nitro alkyl esters would be

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of definite value for cellular oxidation-reduction studies.

Kosolapoff (6) in his review of the chemistry of organophosphorus compounds makes no mention of the nitro alkyl esters. We wish to report our method of preparation of the hitherto unknown tris(2-nitro-2-methylpropyl)-phosphite and our method of preparation of the previously reported (7, 8) tris(2-nitro-2-methylpropyl)-phosphate.

EXPERIMENTAL

Tris(2-nitro-2-methylpropyl)-phosphite.—In a 1,000-ml. two-necked flask equipped with a dropping funnel, magnetic stirrer, and a reflux condenser, were placed 40 Gm. (0.34 mole) of 2-nitro-2-methylpropanol-1[1] dissolved in 200 ml. of dry ether and 24 Gm. (0.30 mole) pyridine. The flask was cooled in an ice bath and 13.8 Gm. (0.1 mole) of phosphorus trichloride diluted with 100 ml. of dry ether was added over a period of three hours with stirring. The resulting mixture was filtered and the filtrate evaporated to dryness under vacuum.

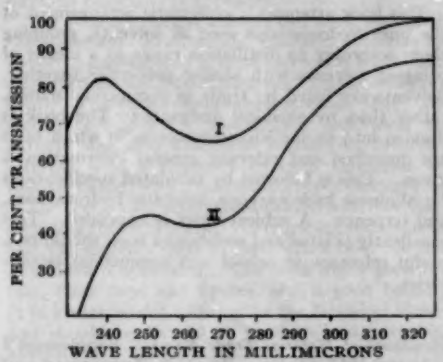

Fig. 1—Ultraviolet spectrum in absolute methanol of phosphate ester 0.002667 mole per liter (I), and phosphite ester 0.006072 mole per liter (II).

The solid residue was extracted with three 200-ml. portions of acetone and the acetone removed under vacuum. The resulting white solid was dissolved in a minimum quantity of acetone and precipitated by adding the acetone solution slowly to an excess of cold water. Recrystallization from the same solvent mixture gave 19 Gm. (50%) of white crystals, insoluble in water, soluble in acetone, ether, absolute alcohol, and melting at 91–93°, uncorrected. The ultraviolet spectrum (Fig. 1) was measured showing a maximum at 276 millimicrons. The molar extinction coefficient was found to be 57.92. A sample dried under high vacuum for eight hours showed the following analysis:

Anal.[2]—Calcd. for $C_{15}H_{33}N_3O_9P$: C, 37.40; H 6.28; N, 10.90; P, 8.04. Found: C, 37.26, 37.34; H, 6.22, 6.28; N, 10.32, 10.49; P, 8.10, 8.17. The phosphite ester was characterized by oxidation to the phosphate derivative using the method of Kessler (3). The resulting crystals, after drying under high vacuum melted at 151.5–152.5°.

Tris(2-nitro-2-methylpropyl)-phosphate.—This compound was prepared in a manner analogous to that described for the phosphite, using redistilled phosphorus oxychloride. After the reaction mixture was worked up in the usual manner, there remained 20 Gm. (50%) of white crystals. The product was insoluble in water, soluble in acetone, absolute alcohol, and melted at 151.5–152.5°, uncorrected. The ultraviolet spectrum (Fig. 1) was measured showing a maximum at 278 millimicrons. The molar extinction coefficient was found to be 65.20. A sample dried under high vacuum for eight hours was used for analysis.

Anal.—Calcd. for $C_{15}H_{34}N_3O_{10}P$: P, 7.72. Found: P, 7.68. A mixed melting point of the permanganate oxidation product of the phosphite and the phosphate prepared as described showed no depression of the melting point.

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[1] Generously supplied by Commercial Solvents Corp.

[2] Carbon, hydrogen, and nitrogen analyses by Clark Microanalytical Laboratory, Urbana, Ill. Phosphorus determination by Parr Bomb-Gravimetric method.

Book Notices

The Neurohypophysis. Edited by H. HELLER. Academic Press Inc., New York, 1957. xv + 275 pp. 19 x 25.5 cm. Price $9.50.

This compilation of the papers and the discussions at the eighth symposium of the Colston Research Society held in 1956 develops the two main lines of progress in the study of the neuropophysis: the evidence that the neurohypophisial hormones are elaborated not in the posterior pituitary lobe, but in the hypothalamus; and the establishment of the chemical nature of the active principles—in some mammalian species. The titles of the papers are: Evidence concerning the endocrine function of the neurohypophysis and its nervous control; Relationship between neurohypophysial structure and function; The comparative aspect of neurosecretion with special reference to the hypothalamo-hypophysial system; The relationship of oxytocin and vasopressin to active proteins of posterior pituitary origin. Studies concerning the existence or non-existence of a single neurohypophysial hormone; Polypeptides with posterior pituitary-like activities; The storage and liberation of neurohypophysial hormones; The metabolism and fate of neurohypophysial hormones; The excretion of posterior pituitary principles in the urine; The effects of hemorrhage and plasma hypertonicity on the neurohypophysis; Polydipsia, antidiuresis and milk ejection caused by hypothalamic stimulation; Conditions under which posterior pituitary hormones increase sodium and potassium excretion by the kidney; The location of antidiuretic action in the mammalian kidney; The antidiuretic action of neurohypophysial hormones in Amphibia; Neurohypophysial hormones and the mammary gland; On oxytocin and uterine function; The release of vasopressin and oxytocin in response to drugs; The adrenal-neurohypophysial relationship; and Comparative aspects of adrenocortical-neurohypophysial relationships. References are given after each paper, but it is too bad that this beautifully printed and bound book does not include a general index as a reference aid. The book will be of particular interest to biochemists, physiologists, and pharmacologists.

Vitamin A. By THOMAS MOORE. D. Van Nostrand Company, Inc., N. J., U. S. distributors for Elsevier Publishing Company, London, 1957. xx + 645 pp. 14.5 x 22.5 cm. Price $14.

This book is presented as a comprehensive review on vitamin A compiled from data obtained by nutritionists, biochemists, chemists, and workers in related fields. The 39 chapters are grouped under the main headings: Historical introduction, Estimation of vitamin A and its provitamins and congeners, Comparative biochemistry and natural history of the carotenoids and vitamin A, Physiology and biochemistry of vitamin A and its provitamins and congeners, Pathology of vitamin A deficiency or excess, Vitamin A in the human, and Special topics. An appendix includes vitamin A deficient diets for animals, determination of carotene in biological materials, and estimation of vitamin A by ultraviolet spectrophotometry. References are given at the end of each chapter, and a very helpful aid in their use is the notation at the bottom of every odd numbered page as to where the next group of references will be found. An author index and a good subject index add to the value of this excellently printed book as an important reference.

Handbook of Solvents. Vol. I: Pure Hydrocarbons. By IRBERT MELLAN. Reinhold Publishing Corporation, New York, 1957. iv + 249 pp. 16 x 23.5 cm. Price $6.50.

This book attempts a systematic arrangement of the pure hydrocarbons used as solvents, grouping them according to distillation range as a means of bringing solvents with similar properties together. Solvents are listed by trade or commercial names, rather than by chemical designation. The book is divided into an introductory section in which tests are described and relevant general information is given. This is followed by tabulated specifications for aliphatic hydrocarbons, aromatic hydrocarbons, and terpenes. A subject index is appended. This excellently printed and well-bound book should be a useful reference in school and commercial laboratories.

Handbook of Material Trade Names. Supplement I to the 1953 edition. By O. T. ZIMMERMAN and IRVIN LAVINE. Industrial Research Service, Inc., Dover, N. H., 1956. x + 383 pp. 19 x 27 cm. Probable price $12.50.

This supplement includes new items and brings up to date the status of many trade names that have been registered as trade-marks since the 1953 edition of this Handbook. The system, style, and format of the supplement are similar to the 1953 edition of the Handbook of Material Trade Names which was reviewed in THIS JOURNAL, 42, 385(1953). Those who have found the earlier volume useful will welcome the issuance of supplement I. Supplement II is being prepared for publication.

Selected Scientific Papers from the Institute Superiore di Sanità (Rome). Vol. 1, Part 1, 1957. Interscience Publishers, Inc., New York, 1957. 233 pp. 17 x 24 cm. Price $8.10.

This book is a compilation of articles of general scientific interest and of particular interest in the field of public health. Subjects include: "The low energy gamma ray spectrum of cosmic radiation," and a section on the genetics and control of insects, particularly the house-fly. Of particular interest to pharmacists is the article on "Chemical and pharmacological studies of the alkaloids of *Strychnos* sp. from Brazil." This paper-back book is beautifully printed and illustrated, but it does not have a general index.

Solvent Extraction in Analytical Chemistry. By GEORGE H. MORRISON and HENRY FREISER. John Wiley & Sons, Inc., New York, 1957. xi + 289 pp. 15 x 23 cm. Price $6.75.

This book covers the theory and practice of solvent extraction methods that are applicable to rapid analyses for trace concentrations and highly complex mixtures of inorganic compounds and materials. The first part of the book takes up Principles of solvent extraction, gives a classification of metal extraction systems and includes chapters on: Formation of metal complexes; Distribution of the extractable species; Chemical interaction in the organic phase; Quantitative treatment of extraction equilibria; and Kinetic factors in extraction. Part 2 includes methods and techniques of extraction. Part 3 covers ion association and chelation in extraction systems, and Part 4 includes selected procedures for the extraction of the elements. A tabulation of organic solvents and their physical constants is appended.

The pharmaceutical analyst can note with relief the statement "Fortunately, emulsion problems are not often encountered in most extraction separations employed for analytical purposes" (in the inorganic field). The sound observation is made that "The method of agitation undoubtedly influences the particle size, and analytical extractions involving solvents with a tendency to form emulsions are best performed with a continuous type of extractor."

The text is thoroughly documented with references including recent reports. An index list the elements and the reagents used in their extraction. A subject index is appended. The book has a good format, clear type and figures, and a good binding. It is a very useful addition to the technical library and should be helpful to teachers and practicing analysts.

Rendiconti Instituto Superiore di Sanità. Vol. XVII, 1954. English Edition of Special Number on Pilot Plant Techniques of Submerged Fermentation. Edited by DOMENICO MAROTTA and E. B. CHAIN. Published under the auspices WHO, 1957, copyright by Fondazione Emanuele Paternó, Rome, 1954. x + 243 pp. 17 x 24 cm. Price: Lit. 5,000.

The text contains twelve chapters entitled: Aeration studies; A laboratory fermenter for vortex and sparger aeration; Fermenters of 90 and 300 L. capacity for vortex and sparger aeration; A "compensated" stuffing box and bearing unit for fermenters of simi-industrial and industrial capacities; Pilot plant for fermentation in submerged culture; A simple rotary shaker; Antifoam agents in aerobic fermentation; Evaluation of the activities of antifoam preparations; Inter-relation of protein and polynucleotide synthesis in *Escherichia coli;* The effect of mechanical agitation on the morphology of *Penicillium chrysogenum* Thom in stirred fermenters; *Romanoa,* a new genus of soil fungus with antibacterial activity; Genetics of *Penicillium chrysogenum.* I Heterokaryosis in *Penicillium chrysogenum;* and Genetics of *Penicillium chrysogenum.* II Segregation and recombination from a heterozygous diploid. Each section begins with a summary of the part of the text (in English, Italian, French, and German versions). The text is in English, with good type and excellent illustrations, but with a paper-back binding. The subject index is hardly more useful than the table of contents for reference purposes, but references are given at the end of each chapter.

Ion Exchangers in Organic and Biochemistry. Edited by CALVIN CALMON and T. R. E. KRESSMAN, Interscience Publishers, New York, 1957. xii + 761 pp. 15 × 23 cm. Price $15.

The editors of this book have attempted to encompass the great increase in knowledge in the field of ion exchange by having various phases of the field covered by 37 authoritative contributors. The book is divided into three parts: the first two dealing with fundamentals and the techniques and apparatus used in the exchange and separation of ions, while the third part deals with applications to specific aspects of organic and biochemistry. Part III covers first, the ion exchange characteristic of body tissue and cells, followed by: organisms, protein materials, special body products and fluids, pharmaceutical and medical applications, plants and plant products, foods, organic chemistry, catalysis, and water.

Particularly interesting to the pharmacy field are the chapters on: Medical and pharmaceutical applications, Isolation and purification of antibiotics, Vitamins, Alkaloids, Nonsteroid hormones, Isolation and analysis of the components of urine, Sodium and potassium removal *in vivo,* and Gastric acidity. The style, type, format, and binding of the book are very good. Each chapter has its references, and author and subject indexes are appended. This book is an excellent addition to the modern scientific library.

Scoville's The Art of Compounding. 9th ed. By GLENN L. JENKINS, DON E. FRANCKE, EDWARD A. BRECHT, and GLEN J. SPERANDIO. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1957. vii + 551 pp. 18 × 25 cm. Price $11.

This excellent book, which is devoted to a clear and comprehensive treatment of pharmaceutical compounding and dispensing, is useful as a textbook for students and as a reference book for practitioners. The authors have avoided the inclusion of material that is readily available in other books and compendia that should be conveniently at hand. This permits full treatment of the selected material without the need for handling an oversize book.

The ninth edition is similar to the eighth edition, which was reviewed in THIS JOURNAL, 40, 362 (1951), with respect to its fine style and format, its clear type and illustrations, and the clarity of its text. A new chapter on ophthalmic solutions has been included and much new material has been added to give the text the new look in modern compounding techniques and formulations. The thorough treatment of incompatibilities is retained as one of the valuable aids for study and reference afforded by this very useful book. It can be recommended without reservation.

British National Formulary. (Standard Edition). Edited by the Joint Formulary Committee of the British Medical Association and The Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1957. 226 pp. 10.5 x 16.5 cm. Price *6s. 6d.*, Interleaved copies 10*s.*

This formulary for prescribers in hospital and general practice is intended also to be useful to pharmacists in Great Britain, particularly as these practices and practitioners are related to the British National Health Service. However, the Joint Formulary Committee notes "that a doctor is free to prescribe what he considers to be in the best interests of his patient, *whether or not the preparation appears in the British National Formulary.*"

The revision of The National Formulary 1955, which was reviewed in THIS JOURNAL, **44**, 319(1955), was made without appreciable change in style or format. Sixty-six preparations were dropped and 101 new preparations have been added. The Committee noted receipt of "evidence of some confusion between the ten-year old National Formulary and a contemporary (69-year old) National Formulary, U. S. A. which is a book of standards," and agreed to change the title to British National Formulary. This was very sporting and should resolve any confusion between the two compendia that are so useful in their respective fields.

The Committee is to be congratulated for continuing to produce a concise compilation of much basic and useful information for the practitioners of medicine and pharmacy.

The British National Formulary, 1957, (Alternative Edition) is similar in many respects to the Standard Edition with the following exceptions: the paragraphs on "Notes for Prescriber" are omitted from the front editions, but are integrated with the information in the formulary section; and the formulary section, which occupies most of the pages in both sections, is arranged in the Alternative Edition according to a pharmacological classification. The Standard Edition "formulary section" is arranged according to a classification by pharmaceutical dosage forms. The price of the Alternative Edition is 7*s. 6d;* interleaved copies, 10.6*d.* 245 pp. 10.5 × 16.5 cm.

The Chemistry and Biology of Purines. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and CECILIA M. O'CONNOR. Little, Brown and Company, Boston, 1957. xii + 327 pp. 13.5 x 20.5 cm. Price $9.

This compilation of papers and discussions includes the following reports: Synthesis and properties of purines of potential biological interest, Some synthetic studies on purines and related heterocycles, Some new *N*-methylpurines, The structure of the hydroxypurines investigated by *O*- and *N*-methylation, The spectra and structure of the monohydroxypurines and other potentially tautomeric purines, the π-electron properties of purine calculated by the L.C.A.O. method, The degradation of uric acid by water under pressure, The 8-position in purines. The chemical and biological transformation of purines into pteridines, *Cyclo*nucleosides, Sterochemistry of nucleoside synthesis, Synthetic chemical investigations related to the metabolism of purines, The effects of potential antipurines on a purine-requiring strain of *Escherichia coli*, The chemistry of new purines in the B_{12} series of vitamins, Biological and microbiological activity of purine analogues of vitamin B_{12}, Puromycin, Chemical and biological behaviors of 9-β-D-ribofuranosylpurine, On the activation of the one-carbon unit for the biosynthesis of purine nucleotides, The enzymatic synthesis of inosinic acid *de novo*, Enzymic control of purines by xanthinc oxidase, The biological effects of 8-azapurines, Biochemical effects of 6-mercaptopurine, and The use of 6-mercatopurine in the treatment of leukemia. References are given after each paper and a subject index is appended. The book should be available to graduate students in the related sciences and should be of particular interest to advanced workers in chemistry and biology.

The Leukemias: Etiology, Pathophysiology, and Treatment. Edited by JOHN W. REBUCK, FRANK H. BETHELL, and RAYMOND W. MONTO. Academic Press Inc., New York, 1957. xii + 711 pp. 15 x 23 cm. Price $13.

This volume is a record of current knowledge regarding leukemic etiology, pathophysiologic changes, leukocytic functions, and therapy of the leukemias. It is a compilation of the reports and discussions of the Henry Ford Hospital international symposium held March, 1956. The material covered is divided under the headings: The Leukemic cell, its structure and antigenicity; Genetic and environmental factors in the transmission of leukemia; Radiation biology of leukocytes; The leukemias and the malignant lymphomas; Leukocytic physiology; Metabolism of amino and nucleic acids in the leukemias; further biochemical considerations in the leukemias; Nucleic acids as the target for chemotherapy: Mechanisms of drug action and resistance.

Particularly interesting to the pharmacist is the final section dealing with the present status of new clinical agents and combinations in treatment of the leukemias. References are given at the end of each paper. Author and subject indexes add to the usefulness of the book as a valuable reference volume. The style, format, type, and binding are excellent and the book should survive the frequent use it will probably be subjected to in many libraries.

The Adrenal Cortex. By I. CHESTER JONES. New York: Cambridge University Press, 1957. x + 315 pp. 14.5 x 22 cm. Price $7.

This monograph gives a comprehensive account of the anatomy and the functions of the adrenal cortex in the different biological species among the vertebrates, with special attention to the mammals and particularly to man. In the 112 pages devoted to the Eutheria (placental mammals), subjects covered are: Gross anatomy, Histology, Adrenal steroid hromones, Biosynthesis of cortical hormones, Control of adrenocortical secretion, Functions of the adrenal cortex, Relationship of the cortex to peripheral tissue, Adrenal-gonad relationships, and Natural abnormalities of function. References are listed alphabetically according to the authors' names, with the title of the article and periodical; and a general index is appended. The book should be of interest to teachers of zoology, histology, embryology, and pharmacology.

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